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HUMANIZED COLLAGEN ANTIBODIES AND RELATED METHODS

by

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HUMANIZED COLLAGEN ANTIBODIES AND RELATED METHODS

BACKGROUND OF THE INVENTION

The present invention relates generally to immunology and more specifically to humanized antibodies and uses thereof.

The extracellular matrix (ECM) plays a fundamental role in the regulation of normal and pathological processes. The most abundantly expressed component found in the ECM is collagen. Triple helical collagen is known to be highly resistant to proteolytic cleavage except by members of the matrix metalloproteinase (MMP) family of enzymes.

Angiogenesis and tumor growth depend on cellular interactions with the extracellular matrix. During angiogenesis and tumor invasion, both endothelial cells as well as tumor cells proteolytically remodel their extracellular microenvironment. The invasive cells then interact with this newly remodeled extracellular matrix followed by migration and invasion. To this end, a major component of the basement membrane surrounding blood vessels is collagen-IV. Moreover, collagen-I is the major component of the interestitial matrix.

One of the major detrimental consequences of the progression of cancer is metastasis beyond the site of the primary tumor. Such metastasis often requires more aggressive therapies, and once metastasis has occurred, the prognosis for survival of a cancer patient decreases dramatically.

The growth of all solid tumors requires new blood vessel growth for continued expansion of the tumors, particularly beyond a minimal size. Because angiogenesis is required for tumor growth, inhibiting angiogenesis is one approach to inhibiting tumor growth. It is therefore desirable to identify molecules that can target angiogenic vasculature. Particularly attractive molecules for targeting angiogenic vasculature are antibodies that can bind specifically to angiogenic vasculature. However, since most antibodies are developed in non-human animals such as mice, these antibodies often have undesirable immunogenic activity that limits their effectiveness for human therapy.

One approach to overcoming the detrimental properties of non-human antibodies is to humanize the antibodies by using human antibody framework region sequences spliced together with the binding domains that confer binding specificity. However, grafting of these binding domains, referred to as complementarity determining regions (CDRs), into human frameworks has often resulted in the loss of binding affinity.

Thus, there exists a need to identify antibodies specific for angiogenic vasculature and to humanize and optimize the antibodies for therapeutic purposes. The following invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a grafted antibody, or functional fragment thereof, comprising one or more

complementarity determining regions (CDRs) having at least one amino acid substitution in one or more CDRs of a heavy chain CDR, where the grafted antibody or functional fragment thereof has specific binding activity for a cryptic collagen epitope. The invention also provides methods of using an antibody having specific binding activity for a cryptic collagen epitope, including methods of inhibiting angiogenesis, tumor growth, and metastasis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequences of primers used to clone nucleic acids encoding HUIV26 and HUI77 antibodies. Figure 1A shows a set of 5' primers for the signal peptide of mouse antibody light chain (SEQ ID NOS: 184-192). Figure 1B shows a set of 5' primers for the signal peptide of mouse antibody heavy chain (SEQ ID NOS: 193-211). Figure 1C shows a set of primers for the constant region of mouse heavy and light chains. Primer 2650 (SEQ ID NO:212) is the 3' primer for mouse kappa light chain constant region (amino acids 123-115). Primer 2656 (SEQ ID NO:213) is the 3' primer for mouse IgM CH1 region (amino acids 121-114). Primer 2706 (SEQ ID NO:214) is the 3' primer for mouse IgM CH1 region (amino acids 131-124).

Figure 2 shows the sequence of the variable region of anti-cryptic collagen site antibody HUIV26. Figure 2A shows the nucleotide sequence of HUIV26 variable region light chain (SEQ ID NO:1). Figure 2B shows the nucleotide sequence of HUIV26 variable region heavy chain (SEQ ID NO:3). Figure 2C shows an alignment

of the amino acid sequence of HUIV26 light chain (V_K) domain of HUIV26 (SEQ ID NO:2) with a human variable region fusion, VKIV/JK2 (SEQ ID NO:6) and an alignment of HUIV26 heavy chain (V_H) domain (SEQ ID NO:4) with a human variable region fusion VHIII/JH6 (SEQ ID NO:8), with CDRs underlined. Amino acids in the framework region that differ between the aligned sequences are indicated by lines.

Figure 3 shows the sequence of the variable region of anti-cryptic collagen site antibody HUI77. Figure 3A shows the nucleotide sequence of HUI77 variable region light chain (SEQ ID NO:9). Figure 3B shows the nucleotide sequence of HUI77 variable region heavy chain (SEQ ID NO:11). Figure 3C shows an alignment of the amino acid sequence of HUI77 light chain (V_K) domain of HUI77 (SEQ ID NO:10) with a human variable region fusion, VKII/JK1 (SEQ ID NO:14) and an alignment of HUI77 heavy chain (V_H) domain (SEQ ID NO:12) with a human variable region fusion VHIII/JH6 (SEQ ID NO:16), with CDRs underlined. Amino acids in the framwork region that differ between the aligned sequences are indicated by lines. Figure 3D shows an alignment of the nucleotide sequence of HUI77 variable region with the sequence of the human framework fusion of DPK13 and JK1 (SEQ ID NO:17).

Figure 4 shows beneficial CDR mutations for anti-cryptic collagen site antibody HUIV26. Figure 4A shows a set of primers used to generate random mutations in LCDR3 and HCDR3 of HUIV26 (HUIV26 LCDR3 primers, SEQ ID NOS:224-232; HUIV26 HCDR3 primers, SEQ ID NOS:233-243). Figure 4B shows a set of primers used to

generate random mutations in LCDR1a (SEQ ID NOS:266-273), LCDR1b (SEQ ID NOS:274-282), LCDR2 (SEQ ID NOS:283-289), HCDR1 (SEQ ID NOS:290-294), HCDR2a (SEQ ID NOS:295-303) and HCDR2b (SEQ ID NOS:304-311) of HUIV26. Figure 4C shows beneficial CDR mutations of the HUIV26 antibody.

Figure 5 shows beneficial CDR mutations for anti-cryptic collagen site antibody HUI77. Figure 5A shows a set of primers used to generate random mutations in LCDR3 and HCDR3 of HUI77. Figure 5B shows a set of primers used to generate random mutations in LCDR1a (SEQ ID NOS:312-319), LCDR1b (SEQ ID NOS:320-327), LCDR2 (SEQ ID NOS:328-334), HCDR1 (SEQ ID NOS:335-341), HCDR2a (SEQ ID NOS:342-349) and HCDR2b (SEQ ID NOS:350-357) of HUI77. Figure 5C shows beneficial CDR mutations of the HUI77 antibody.

Figure 6 shows mutations in combinatorial variants of the HUIV26 antibody. The position of amino acids are shown, with mutations different than wild type shown in bold. The relative activity of combinatorial variants is shown as "$SPEK_{on}$" and "$SPEK_{off}$" (last column). Primers used to create the combinatorial libraries are also shown (SEQ ID NOS:163-173).

Figure 7 shows mutations in combinatorial variants of the HUI77 antibody. The position of amino acids are shown, with mutations different than wild type shown in bold. The relative activity of combinatorial variants is shown as "$SPEK_{on}$" and "$SPEK_{off}$" (last column). Primers used to create the combinatorial libraries are also shown (SEQ ID NOS:174-183).

Figure 8 shows the activity and specificity of HUIV26 variants. The binding of purified Fabs of IX-IV26, containing wild type HUIV26 CDRs, and the HUIV26 variants 2D4H1-C3 and DhuG5 is shown for denatured collagen IV (Figure 8A), denatured collagen I (Figure 8B) and native collagen IV (Figure 8C).

Figure 9 shows the activity and specificity of HUI77 variants. The binding of purified Fabs of IX-I77, containing wild type HUI77 CDRs, and HUI77 variants Qh2b-B7 and QhuD9 is shown for denatured collagen I (Figure 9A), denatured collagen IV (Figure 9B) and native collagen I (Figure 9C).

Figure 10 shows the binding activity of the HUIV26 variant DhuH8. The binding activity of the Fab form and the IgG form of the antibody to denatured (d-IV) and native (n-IV) human collagen IV is shown.

Figure 11 shows the effect of the HUI77 variant QH2b on B16 melanoma cell proliferation. B16 melanoma cells grown in culture were not treated (control; squares) or treated with the IgG form of the QH2b variant (circles).

DETAILED DESCRIPTION OF THE INVENTION

The invention provides antibodies specific for a cryptic collagen site, which is exposed during angiogenesis and tumor cell invasion through collagenous tissue and thus serves as an antibody that can target angiogenic vasculature. The antibodies are optimized for binding activity to a cryptic collagen site. The

antibodies can be used to target angiogenic vasculature for diagnostic or therapeutic purposes. The antibodies can also be used to inhibit tumor growth.

As used herein, the term "CDR" or "complementarity determining region" is intended to mean the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. These particular regions have been described by Kabat et al., J. Biol. Chem. 252:6609-6616 (1977); Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of proteins of immunological interest" (1991); by Chothia et al., J. Mol. Biol. 196:901-917 (1987); and MacCallum et al., J. Mol. Biol. 262:732-745 (1996), where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or grafted antibodies or variants thereof is intended to be within the scope of the term as defined and used herein. The amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison.

Table 1: CDR Definitions

	Kabat[1]	Chothia[2]	MacCallum[3]
V_H CDR1	31-35	26-32	30-35
V_H CDR2	50-65	53-55	47-58
V_H CDR3	95-102	96-101	93-101
V_L CDR1	24-34	26-32	30-36
V_L CDR2	50-56	50-52	46-55
V_L CDR3	89-97	91-96	89-96

[1] Residue numbering follows the nomenclature of Kabat et al., *supra*

[2] Residue numbering follows the nomenclature of Chothia et al., *supra*

[3] Residue numbering follows the nomenclature of MacCallum et al., *supra*

As used herein, the term "framework" when used in reference to an antibody variable region is intended to mean all amino acid residues outside the CDR regions within the variable region of an antibody. A variable region framework is generally between about 100-120 amino acids in length but is intended to reference only those amino acids outside of the CDRs. As used herein, the term "framework region" is intended to mean each domain of the framework that is separated by the CDRs.

As used herein,the term "donor" is intended to mean a parent antibody molecule or fragment thereof from which a portion is derived from, given to or contributes to another antibody molecule or fragment thereof so as to confer either a structural or functional characteristic of the parent molecule onto the receiving molecule. For

the specific example of CDR grafting, the parent molecule from which the grafted CDRs are derived is a donor molecule. The donor CDRs confer binding affinity of the parent molecule onto the receiving molecule. The donor molecule can be a different species or the same species as the receiving molecule. If the donor and receiving molecules are of the same species, it is understood that it is sufficient that the donor is a separate and distinct molecule from the receiving molecule.

As used herein, the term "acceptor" is intended to mean an antibody molecule or fragment thereof which is to receive the donated portion from the parent or donor antibody molecule or fragment thereof. An acceptor antibody molecule or fragment thereof is therefore imparted with the structural or functional characteristic of the donated portion of the parent molecule. For the specific example of CDR grafting, an acceptor molecule, including framework and/or other antibody fragments, is the receiving molecule into which the CDRs are grafted. The acceptor antibody molecule or fragment is imparted with the binding affinity of the donor CDRs or parent molecule. As with a donor molecule, it is understood that an acceptor molecule can be the same or a different species as the donor.

A "variable region" when used in reference to an antibody or a heavy or light chain thereof is intended to mean the amino terminal portion of an antibody which confers antigen binding onto the molecule and which is not the constant region. The term is intended to include functional fragments thereof which maintain some of all of the binding function of the whole variable region.

Therefore, the term "heteromeric variable region binding fragments" is intended to mean at least one heavy chain variable region and at least one light chain variable regions or functional fragments thereof assembled into a heteromeric complex. Heteromeric variable region binding fragments include, for example, functional fragments such as Fab, $F(ab)_2$, Fv, single chain Fv (scFv) and the like. Such functional fragments are well known to those skilled in the art. Accordingly, the use of these terms in describing functional fragments of a heteromeric variable region is intended to correspond to the definitions well known to those skilled in the art. Such terms are described in, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989); Molec. Biology and Biotechnology: A Comprehensive Desk Reference (Myers, R.A. (ed.), New York: VCH Publisher, Inc.); Huston et al., Cell Biophysics, 22:189-224 (1993); Plückthun and Skerra, Meth. Enzymol., 178:497-515 (1989); and in Day, E.D., Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc., New York, NY (1990).

As used herein, the term "population" is intended to refer to a group of two or more different molecules. A population can be as large as the number of individual molecules currently available to the user or able to be made by one skilled in the art. Populations can be as small as 2-4 molecules or as large as 10^{13} molecules. Generally, a population will contain two or more, three or more, five or more, nine or more, ten or more, twelve or more, fifteen or more, or twenty or more different molecules. A population can also contain tens or hundreds of different molecules or even thousands of

different molecules. For example, a population can contain about 20 to about 100,000 different molecules or more, for example about 25 or more, 30 or more, 40 or more, 50 or more, 75 or more, 100 or more, 150 or more, 200 or more, 300 or more, 500 or more, or 1000 or more different molecules, and can contain 10,000, 100,000 or even $1x10^6$ or more different molecules. Those skilled in the art will know what size and diversity of a population is suitable for a particular application.

As used herein, the term "altered" when used in reference to an antibody variable region is intended to mean a heavy or light chain variable region that contains one or more amino acid changes in a framework region, a CDR or both compared to the parent amino acid sequence at the same position. Where an altered variable region is derived from or composed of donor and acceptor regions, the changed amino acid residues within the altered species are to be compared to their respective amino acid positions within the parent donor and acceptor regions.

As used herein, the term "nucleic acid" or "nucleic acids" is intended to mean a single- or double-stranded DNA or RNA molecule. A nucleic acid molecule of the invention can be of linear, circular or branched configuration, and can represent either the sense or antisense strand, or both, of a nucleic acid molecule. The term also is intended to include nucleic acid molecules of both synthetic and natural origin. A nucleic acid molecule of natural origin can be derived from any animal, such as a human, non-human primate, mouse, rat, rabbit, bovine, porcine, ovine, canine, feline, or amphibian, or from a lower eukaryote, such as

Drosophila, *C. elegans*, yeast, and the like. A synthetic nucleic acid includes, for example, chemical and enzymatic synthesis. The term "nucleic acid" or "nucleic acids" is similarly intended to include analogues of natural nucleotides which have similar functional properties as the referenced nucleic acid and which can be utilized in a manner similar to naturally occurring nucleotides and nucleosides.

As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. An antibody useful in the invention, or antigen binding fragment of such an antibody, is characterized by having specific binding activity for a polypeptide or a peptide portion thereof of at least about 1 x 10^5 M^{-1}. Thus, Fab, $F(ab')_2$, Fd, Fv, single chain Fv (scFv) fragments of an antibody and the like, which retain specific binding activity for a polypeptide, are included within the definition of an antibody. Specific binding activity of an antibody for a polypeptide can be readily determined by one skilled in the art, for example, by comparing the binding activity of an antibody to a particular polypeptide versus a control polypeptide that is not the particular polypeptide. Methods of preparing polyclonal or monoclonal antibodies are well known to those skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988)).

In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as

non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al. (Science 246:1275-1281 (1989)). These and other methods of making functional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Harlow and Lane, *supra*, 1988); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995)).

As used herein, specific binding means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity, for example, an antibody that binds a distinct epitope or antigen. Specificity of binding also can be determined, for example, by competition with a control molecule, for example, competition with an excess of the same molecule. In this case, specific binding is indicated if the binding of a molecule is competitively inhibited by itself. Thus, specific binding between an antibody and antigen is measurably different from a

non-specific interaction and occurs via the antigen binding site of the antibody.

As used herein, selective binding refers to a binding interaction that is both specific and discriminating between molecules, for example, an antibody that binds to a single molecule or closely related molecules. For example, an antibody can exhibit specificity for an antigen that can be both specific and selective for the antigen if the epitope is unique to a molecule. Thus, a molecule having selective binding can differentiate between molecules, as exemplified by an antibody having specificity for an epitope unique to one molecule or closely related molecules. Alternatively, an antibody can have specificity for an epitope that is common to many molecules, for example, a carbohydrate that is expressed on a number of molecules. Such an antibody has specific binding but is not selective for one molecule or closely related molecules.

As used herein the term "binding affinity" is intended to mean the strength of a binding interaction and includes both the actual binding affinity as well as the apparent binding affinity. The actual binding affinity is a ratio of the association rate over the disassociation rate. Therefore, conferring or optimizing binding affinity includes altering either or both of these components to achieve the desired level of binding affinity. The apparent affinity can include, for example, the avidity of the interaction. For example, a bivalent heteromeric variable region binding fragment can exhibit altered or optimized binding affinity due to its valency.

As used herein, the term "substantially the same" when used in reference to binding affinity is intended to mean similar or identical binding affinities where one molecule has a binding affinity that is similar to another molecule within the experimental variability of the affinity measurement. The experimental variability of the binding affinity measurement is dependent upon the specific assay used and is known to those skilled in the art.

As used herein, the term "optimizing" when used in reference to a variable region or a functional fragment thereof is intended to mean that the functional activity of the variable region has been modified compared to the activity of a parent variable region or a donor variable region, resulting in a desirable change in activity. A variable region or functional fragment thereof exhibiting optimized activity can exhibit, for example, higher affinity or lower affinity binding, or increased or decreased association or dissociation rates compared to an unaltered variable region. A variable region or functional fragment thereof exhibiting optimized activity also can exhibit increased stability such as increased half-life in a particular organism. For example, an antibody activity can be optimized to increase stability by decreasing susceptibility to proteolysis. An antibody exhibiting optimized activity also can exhibit lower affinity binding, including decreased association rates or increased dissociation rates, if desired. An optimized variable region exhibiting lower affinity binding is useful, for example, for penetrating a solid tumor. In contrast to a higher affinity variable region, which would bind to the

peripheral regions of the tumor but would be unable to penetrate to the inner regions of the tumor due to its high affinity, a lower affinity variable region would be advantageous for penetrating the inner regions of the tumor. As with optimization of binding affinities above, optimization of a catalytic variable region can be, for example, increased or decreased catalytic rates, disassociation constants or association constants.

As used herein, a "cryptic collagen site" or "cryptic collagen epitope" refers to an epitope of a collagen molecule that is less accessible to binding of an antibody, or functional fragment thereof, in native collagen than in denatured collagen. An antibody having binding activity for a cryptic collagen epitope preferentially recognizes denatured collagen over native collagen, that is, has a higher binding affinity for denatured over native collagen. For example, such an antibody can have at least about a 2-fold or greater preference, that is, at least about 2-fold higher binding activity, for denatured collage over native collagen, and can exhibit about a 3-fold or greater preference, about a 5-fold or greater preference, about a 10-fold or greater preference, about a 15-fold or greater preference, about a 20-fold or greater preference, about a 25-fold or greater preference, about a 50-fold or greater preference, about a 100-fold or greater preference, or even a higher preference for denatured over native collagen.

Native collagen herein refers to a molecule where three alpha-chains are organized in a triple helical molecule. Native collagen can be of different

stages of post-translational processing such as pro-collagen and any intermediates in the generation of a mature tissue form of collagen, or collagen molecules isolated by limited proteolysis of tissues under conditions where the triple-helical structure of collagen is not disrupted. Thus, native collagen can be an intact collagen molecule or can contain non-triple-helical sequences flanking triple-helical regions, so long as the triple-helical is not disrupted. Denatured collagen herein refers to collagen where the triple helix is completely or partially disrupted such that a cryptic epitope is made accessible. Denaturation of collagen can occur *in situ* by the action of proteinases, for example, matrix metalloproteinases, that cleave collagen within triple helical regions, rendering the resulting fragments of the triple helix unstable. Denaturation of collagen can be induced *in vitro* by thermal or chemical denaturation of native collagen. Denatured collagen can also be prepared *in vitro* by treatment of native collagen with proteinases capable of cleaving a triple helical region(s), which are commonly referred to as collagenolytic enzymes, at temperatures where the resulting fragments of the triple helix are thermally unstable. Denatured collagen can be obtained by denaturation of native collagens of different stages of post-translational processing or denaturation of native collagen isolated from tissues by limited proteolysis. One skilled in the art will know a variety of methods for isolation of native collagens and a variety of methods to denature a triple helix that contains a cryptic collagen epitope.

An antibody of the invention can have binding activity for a cryptic collagen epitope that is the same as the respective parental mouse antibody. For example, an antibody of the invention having CDRs derived from HUIV26 can have essentially the same binding specificity as the mouse HUIV26 antibody described by Xu et al., Hybridoma 19:375-385 (2000); Xu et al., J. Cell Biol. 154:1069-1079 (2001); and WO 00/40597, each of which is incorporated herein by reference. Similarly, an antibody of the invention having CDRs derived from HUI77 can have essentially the same binding specificity as the mouse HUI77 antibody described by Xu et al., *supra*, 2000; Xu et al., *supra*, 2001; and WO 00/40597. Such binding specificity can be tested by the methods disclosed herein, for example, by comparing the activity of an antibody of the invention to the corresponding parental mouse antibody. For example, an antibody of the invention derived from HUIV26 can be compared to a corresponding mouse antibody having the variable region amino acid sequence shown in Figure 2C (SEQ ID NOS:2 and 4). Similarly, an antibody of the invention derived from HUI77 can be compared to a corresponding mouse antibody having the variable region amino acid sequence shown in Figure 3C (SEQ ID NOS:10 and 12). Similar binding specificity can be determined, for example, by competitive binding with the corresponding parental antibody. It is understood that an antibody of the invention can have essentially the same specificity as the corresponding parental antibody or can have altered specificity so long as the antibody has binding activity for a cryptic collagen epitope.

The invention provides antibodies having specific binding activity for a cryptic collagen epitope. The antibodies contain at least one CDR having at least one amino acid substitution in a CDR of the antibodies HUIV26 and HUI77, which are antibodies that bind to a cryptic collagen site. The invention also provides nucleic acids encoding these antibodies. The invention further provides methods using the antibodies.

Highly specific monoclonal antibodies have been developed that recognize a cryptic domain of human collagen, designated HUIV26 and HUI77 (see Xu et al., Hybridoma 19:375-385 (2000); Xu et al., J. Cell Biol. 154:1069-1079 (2001); WO 00/40597, each of which is incorporated herein by reference). Monoclonal antibody HUIV26 recognizes a cryptic domain of human collagen-IV, and HUI77 recognizes a cryptic domain of human collagen-I and IV that is also common to collagens II, III and V. This cryptic domain(s) is less accessible under most normal physiological conditions but becomes accessible following proteolytic remodeling of the collagen triple helix *in vivo*. Thus, cryptic collagen epitope(s) can become more accessible during invasive cellular processes. Importantly, the cryptic domain(s) defined by these antibodies was shown to be exposed within the basement membrane of tumor associated angiogenic blood vessels from human tumors including, breast, bladder and melanoma tumors. However, this cryptic domain was less exposed within the vessels or normal tissues tested. Therefore, the antibodies HUIV26 and HUI77 represent important and specific markers of angiogenic blood vessels. These cryptic domain(s) plays an important role in regulating angiogenesis and tumor growth since the

monoclonal antibodies HUIV26 and HUI77 potently inhibit angiogensis and human tumor growth in the chick embryo, rat and mouse models following systemic administration (Xu et al., *supra*, 2001). Thus, these monoclonal antibodies and the antibodies of the invention having specific binding activity for these cryptic collagen site(s) represent a highly potent and effective new therapeutic reagent for the treatment for diseases characterized by aberrant neovascularization.

A nucleic acid sequence of the invention can include a sequence that is the same or substantially the same as a specifically recited SEQ ID NO. Similarly, an amino acid sequence of the invention can include a sequence that is the same or substantially the same as a specifically recited SEQ ID NO. As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to a reference sequence, for example, the sequence of a parent antibody. Such a considerable degree, amount or extent of sequence identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known. Thus, a nucleotide sequence which is substantially the same nucleotide sequence as a heavy or light chain of an antibody of the invention, including fragments thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as encoding or as being the amino acid sequence as the parent antibody sequence. Minor modifications thereof are included so

long as they are recognizable as a parent antibody sequence. Similarly, an amino acid sequence which is substantially the same amino acid sequence as a heavy or light chain of an antibody of the invention, or functional fragment thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as representing the amino acid sequence of parent antibody and minor modifications thereof. When determining whether a nucleotide or amino acid sequence is substantially the same as a parent antibody, consideration is given to the number of changes relative to the parent antibody together with whether the function is maintained, for example, whether the function of binding to a cryptic collagen site is maintained for antibodies of the invention.

Minor modification of these nucleotide sequences and/or amino acids are intended to be included as heavy and light chain encoding nucleic acids and their functional fragments. Such minor modifications include, for example, those which do not change the encoded amino acid sequence due to the degeneracy of the genetic code as well as those which result in only a conservative substitution of the encoded amino acid sequence. Conservative substitutions of encoded amino acids include, for example, amino acids which belong within the following groups: (1) non-polar amino acids (Gly, Ala, Val, Leu, and Ile); (2) polar neutral amino acids (Cys, Met, Ser, Thr, Asn, and Gln); (3) polar acidic amino acids (Asp and Glu); (4) polar basic amino acids (Lys, Arg and His); and (5) aromatic amino acids (Phe, Trp, Tyr, and His). Other minor modifications are included within the nucleic acids encoding heavy and light chain

polypeptides of the invention so long as the nucleic acid or encoded polypeptides retain some or all of their function as described herein.

To generate antibodies of the invention having specific binding activity for a cryptic collagen epitope, the heavy and light chain variable regions of the antibodies HUIV26 and HUI77 were cloned and sequenced (see Example I and Figures 2 and 3). CDRs of the heavy and light chain variable regions were identified. Exemplary heavy and light chain CDRs, as determined by the numbering of Kabat, are shown in Figures 2C and 3C (underlined). Exemplary heavy and light chain CDRs of HUIV26 include, for example, V_L CDR1, KSSQSLLNSGNQKNYLA (SEQ ID NO:20); V_L CDR2, GASTRES (SEQ ID NO:22); V_L CDR3, QNDHSYPYT (SEQ ID NO:24); V_H CDR1, GFDFSRYWMS (SEQ ID NO:26); V_H CDR2, EINPDSSTINYTPSLKD (SEQ ID NO:28); and V_H CDR3, PVDGYYDAMDY (SEQ ID NO:30). Exemplary heavy and light chain CDRs of HUI77 include, for example, V_L CDR1, RSSQSIVHSNGNTYLE (SEQ ID NO:32); V_L CDR2, KVSNRFS (SEQ ID NO:34); V_L CDR3, FQGSHVPWT (SEQ ID NO:36); V_H CDR1, GFSLSTSGMGVG (SEQ ID NO:38); V_H CDR2, DIWWDDNKYYNPSLKS (SEQ ID NO:40); and V_H CDR3, RANYGNPYYAMDY (SEQ ID NO:42).

Libraries of CDR variants containing single amino acid substitutions were generated (Example II). The libraries were screened for binding to a cryptic collagen site, and single amino acid mutations having beneficial activity were identified. Combinatorial mutants, in which two or more variant CDRs containing at least one amino acid substitution relative to parental HUIV26 or HUI77 CDRs were combined and screened for

activity (Example III). A number of combinatorial mutants having optimized activity for binding to a cryptic collagen site were identified.

The antibodies of the invention having binding activity for a cryptic collagen epitope. As disclosed herein, the collagen can be denatured by any of a variety of methods so long as an antigenic determinant is exposed that was less accessible in native collagen. Such methods include, for example, proteolytic digestion, heat or thermal denaturation, chemical denaturation, and the like. One skilled in the art will know a variety of methods suitable for denaturing a collagen molecule to reveal a cryptic collagen site or epitope. Furthermore, the method of denaturation can be a combination of two or more denaturation methods, for example, proteolytic digestion combined with chemical and/or thermal denaturation. For example, proteolytic digestion can be used to cleave collagen, resulting in a collagen molecule that is more susceptible to thermal or chemical denaturation. An exemplary protease that can be used to denature collagen is matrix metalloproteinase, which can be used *in vitro* and can function *in vivo* to cleave collagen within triple helical regions and at body temperature in a mammal.

The invention provides grafted antibodies of the HUIV26 and HUI77 antibodies. In one embodiment, the invention provides a grafted antibody of HUIV26. The grafted antibody, or functional fragment thereof, comprises one or more complementarity determining regions (CDRs) having at least one amino acid substitution in one or more CDRs of a heavy chain CDR selected from the group

consisting of SEQ ID NOS:26, 28 and 30 or a light chain CDR selected from the group consisting of SEQ ID NOS:20, 22 and 24, the grafted antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

In another embodiment, the invention provides a grafted antibody of HUI77. The grafted antibody, or functional fragment thereof, comprises one or more complementarity determining regions (CDRs) having at least one amino acid substitution in one or more CDRs of a heavy chain CDR selected from the group consisting of SEQ ID NOS:38, 40 and 42 or a light chain CDR selected from the group consisting of SEQ ID NOS:32, 34 and 36, the grafted antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

The invention additionally provides antibodies, or functional fragments thereof, containing specifically recited CDRs, where the antibody or functional fragment thereof has specific binding activity for a cryptic collagen epitope. Such antibodies include those having at least a single amino acid substitution and which retain binding activity for a cryptic collagen epitope. Included among such CDR variants are those described in Figures 4 and 5.

Exemplary CDRs of the invention having a single amino acid substitution in a CDR of HUIV26 include, for example, those described below, in which the position of the amino acid mutation in the numbering of Kabat is indicated along with the amino acid substitution from

wild type to mutant (wild type→mutant). Such exemplary CDRs include HuIV26 V_H CDR1 31R→H (SEQ ID NO:43); HuIV26 V_H CDR1 34M→I (SEQ ID NO:44); HuIV26 V_H CDR1 35S→T (SEQ ID NO:45); HuIV26 V_H CDR1 35S→A (SEQ ID NO:46); HuIV26 V_H CDR1 35S→G (SEQ ID NO:47); HuIV26 V_H CDR2 57I→A (SEQ ID NO:48); HuIV26 V_H CDR2 57I→S (SEQ ID NO:49); HuIV26 V_H CDR2 62S→Y (SEQ ID NO:50); HuIV26 V_H CDR2 62S→A (SEQ ID NO:51); HuIV26 V_H CDR2 62S→H (SEQ ID NO:52); HuIV26 V_H CDR2 62S→G (SEQ ID NO:53); HuIV26 V_H CDR2 64K→Q (SEQ ID NO:54); HuIV26 V_H CDR2 65D→S (SEQ ID NO:55); HuIV26 V_H CDR3 97D→P (SEQ ID NO:56); HuIV26 V_H CDR3 97D→G (SEQ ID NO:57); HuIV26 V_H CDR3 97D→T (SEQ ID NO:58); HuIV26 V_H CDR3 97D→A (SEQ ID NO:59); HuIV26 V_H CDR3 98G→P (SEQ ID NO:60); HuIV26 V_H CDR3 98G→A (SEQ ID NO:61); HuIV26 V_H CDR3 98G→H (SEQ ID NO:62); HuIV26 V_H CDR3 102Y→P (SEQ ID NO:63); HuIV26 V_H CDR3 102Y→N (SEQ ID NO:64); HuIV26 V_L CDR1 27Q→R (SEQ ID NO:65); HuIV26 V_L CDR1 27Q→S (SEQ ID NO:66); HuIV26 V_L CDR1 27dN→S (SEQ ID NO:67); HuIV26 V_L CDR1 27eS→Y (SEQ ID NO:68); HuIV26 V_L CDR1 27eS→W (SEQ ID NO:69); HuIV26 V_L CDR1 27eS→H (SEQ ID NO:70); HuIV26 V_L CDR1 27eS→R (SEQ ID NO:71); HuIV26 V_L CDR1 27fG→Y (SEQ ID NO:72); HuIV26 V_L CDR1 27fG→R (SEQ ID NO:73); HuIV26 V_L CDR1 27fG→H (SEQ ID NO:74); HuIV26 V_L CDR1 27fG→I (SEQ ID NO:75); HuIV26 V_L CDR1 29Q→K (SEQ ID NO:76); HuIV26 V_L CDR3 93S→Q (SEQ ID NO:77); HuIV26 V_L CDR3 93S→G (SEQ ID NO:78); HuIV26 V_L CDR3 93S→L (SEQ ID NO:79); HuIV26 V_L CDR3 93S→A (SEQ ID NO:80); HuIV26 V_L CDR3 93S→T (SEQ ID NO:81); HuIV26 V_L CDR3 93S→V (SEQ ID NO:82); HuIV26 V_L CDR3 94Y→N (SEQ ID NO:83); HuIV26 V_L CDR3 94Y→S (SEQ ID NO:84); HuIV26 V_L CDR3 94Y→P (SEQ ID NO:85); HuIV26 V_L CDR3 94Y→M (SEQ ID NO:86); and HuIV26 VL CDR2 57I→V (SEQ ID NO:162).

Exemplary CDRs of the invention having a single amino acid substitution in a CDR of HUI77 include, for example, those described below, in which the position of the amino acid mutation in the numbering of Kabat is indicated along with the amino acid substitution from wild type to mutant (wild type→mutant). Such exemplary CDRs include HUI77 V_H CDR1 32S→P (SEQ ID NO:87); HUI77 V_H CDR1 32S→W (SEQ ID NO:88); HUI77 V_H CDR1 35bG→W (SEQ ID NO:89); HUI77 V_H CDR1 35bG→L (SEQ ID NO:90); HUI77 V_H CDR1 35bG→A (SEQ ID NO:91); HUI77 V_H CDR2 59Y→S (SEQ ID NO:92); HUI77 V_H CDR2 59Y→A (SEQ ID NO:93); HUI77 V_H CDR2 59Y→P (SEQ ID NO:94); HUI77 V_H CDR2 64K→P (SEQ ID NO:95); HUI77 V_H CDR3 95R→P (SEQ ID NO:96); HUI77 V_H CDR3 95R→Q (SEQ ID NO:97); HUI77 V_H CDR3 95R→L (SEQ ID NO:98); HUI77 V_H CDR3 95R→T (SEQ ID NO:99); HUI77 V_H CDR3 95R→V (SEQ ID NO:100); HUI77 V_H CDR3 100N→V (SEQ ID NO:101); HUI77 V_H CDR3 100N→W (SEQ ID NO:102); HUI77 V_H CDR3 100eM→Q (SEQ ID NO:103); HUI77 V_H CDR3 100eM→N (SEQ ID NO:104); HUI77 V_H CDR3 100eM→T (SEQ ID NO:105); HUI77 V_H CDR3 102Y→K (SEQ ID NO:106); HUI77 V_H CDR3 102Y→T (SEQ ID NO:107); HUI77 V_H CDR3 102Y→M (SEQ ID NO:108); HUI77 V_H CDR3 102Y→H (SEQ ID NO:109); HUI77 V_L CDR1 27cV→P (SEQ ID NO:110); HUI77 V_L CDR1 27cV→W (SEQ ID NO:111); HUI77 V_L CDR1 27dH→L (SEQ ID NO:112); HUI77 V_L CDR1 27dH→S (SEQ ID NO:113); HUI77 V_L CDR1 27eS→W (SEQ ID NO:114); HUI77 V_L CDR1 28N→Y (SEQ ID NO:115); HUI77 V_L CDR1 28N→W (SEQ ID NO:116); HUI77 V_L CDR1 30N→Y (SEQ ID NO:117); HUI77 V_L CDR1 33L→F (SEQ ID NO:118); HUI77 V_L CDR1 33L→V (SEQ ID NO:119); HUI77 V_L CDR2 50K→S (SEQ ID NO:120); HUI77 V_L CDR2 51V→A (SEQ ID NO:121); HUI77 V_L CDR2 53N→S (SEQ ID NO:122); HUI77 V_L CDR2 54R→L (SEQ ID NO:123); HUI77 V_L CDR2 56S→W (SEQ ID NO:124); HUI77 V_L CDR2 56S→F (SEQ ID NO:125); HUI77 V_L CDR3 89F→V (SEQ ID NO:126); HUI77 V_L CDR3 89F→H (SEQ ID

NO:127); HUI77 V_L CDR3 90Q→R (SEQ ID NO:128); HUI77 V_L CDR3 90Q→W (SEQ ID NO:129); HUI77 V_L CDR3 91G→S (SEQ ID NO:130); HUI77 V_L CDR3 92S→W (SEQ ID NO:131); HUI77 V_L CDR3 92S→E (SEQ ID NO:132); HUI77 V_L CDR3 93H→L (SEQ ID NO:133); HUI77 V_L CDR3 93H→T (SEQ ID NO:134); HUI77 V_L CDR3 93H→S (SEQ ID NO:135); HUI77 V_L CDR3 93H→A (SEQ ID NO:136); HUI77 V_L CDR3 93H→Q (SEQ ID NO:137); HUI77 V_L CDR3 94V→T (SEQ ID NO:138); HUI77 V_L CDR3 97T→A (SEQ ID NO:139); HUI77 V_L CDR3 97T→R (SEQ ID NO:140); HUI77 V_L CDR3 97T→H (SEQ ID NO:141); HUI77 V_L CDR3 97T→K (SEQ ID NO:142); HUI77 V_L CDR3 97T→I (SEQ ID NO:143); HUI77 V_H CDR2 59Y→T (SEQ ID NO:144); HUI77 V_L CDR3 94V→F (SEQ ID NO:145); and HUI77 V_L CDR1 28N→Q (SEQ ID NO:146).

In addition to CDRs having single amino acid substitutions, the invention additionally provides HUIV26 and HUI77 CDRs having two or more amino acid substitutions. Exemplary CDRs having two or more amino acid substitutions in HUIV26 include, for example, HUIV26 V_H CDR2 57I→A/62S→A (SEQ ID NO:154); HUIV26 V_H CDR2 57I→A/62S→Y (SEQ ID NO:155); HUIV26 V_H CDR2 57I→A/62S→H (SEQ ID NO:156); HUIV26 V_L CDR1 27eS→W/27fG→Y (SEQ ID NO:157); HUIV26 V_L CDR1 27eS→Y/27fG→Y (SEQ ID NO:158); HUIV26 V_L CDR1 27eS→Y/27fG→H (SEQ ID NO:159); HUIV26 V_L CDR1 27eS→R/27fG→Y (SEQ ID NO:160); and HUIV26 V_L CDR1 27eS→W/27fG→H (SEQ ID NO:161) (see Figure 6). Exemplary CDRs having two or more amino acid substitutions in HUI77 include, for example, HUI77 V_H CDR1 32S→P/35bG→W (SEQ ID NO:147); HUI77 V_H CDR1 32S→P/35bG→A (SEQ ID NO:148); HUI77 V_L CDR1 27dH→S/28N→W (SEQ ID NO:149); HUI77 V_L CDR1 27dH→S/28N→Y (SEQ ID NO:150); HUI77 V_L CDR1 27dH→S/28N→Q (SEQ ID NO:151); HUI77 V_L CDR1 28N→Q/33L→F (SEQ ID NO:152); HUI77 V_L CDR1 27H→S/28N→W/33L→F (SEQ ID NO:153);

and HUI77 V_L CDR3 91G-S/94V-F (SEQ ID NO:358) (see Figure 7).

The invention provides an antibody having at least one of the above variant CDR sequences. It is understood that any combination of HUIV26 CDRs can be combined with mutant and/or wild type CDRs to generate an HUIV26 grafted antibody, so long as binding activity to a cryptic collagen site is maintained. Similarly, any combination of HUI77 CDRs can be combined with mutant and/or wild type CDRs to generate a HUI77 grafted antibody so long as binding activity to a cryptic collagen site is maintained. Thus, any combination of single amino acid substitutions can be combined with other CDR mutants to generate an antibody having at least two variant CDRs. Furthermore, any single mutation at different positions within the same CDR can be combined to generate a CDR having 2 or more amino acid substitutions at two or more positions. Any of the single or multiple mutations can be combined so long as binding activity to a cryptic collagen site is maintained.

Thus, the invention provides an antibody, or functional fragment thereof, comprising one or more CDRs selected from the group consisting of CDRs referenced as SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70,

SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78; SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, SEQ ID NO:160, SEQ ID NO:161, and SEQ ID NO:162, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

The invention additionally provides an antibody, or functional fragment thereof, comprising one or more CDRs selected from the group consisting of CDRs referenced as SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:120, SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123, SEQ ID NO:124, SEQ ID NO:125, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:131, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, SEQ ID NO:149, SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, and SED ID NO:358 the antibody or functional

fragment thereof having specific binding activity for a cryptic collagen epitope.

The invention further provides an antibody, or functional fragment thereof, comprising a heavy chain polypeptide comprising one or more CDRs having at least one amino acid substitution in one or more heavy chain CDRs, the heavy chain CDRs selected from the group consisting of a heavy chain CDR1 selected from the group consisting of CDRs referenced as SEQ ID NOS:26, 43, 44, 45, 46, and 47; a heavy chain CDR2 selected from the group consisting of CDRs referenced as SEQ ID NOS:28, 48, 49, 50, 51, 52, 53, 54, and 55; and a heavy chain CDR3 selected from the group consisting of CDRs referenced as SEQ ID NOS:30, 56, 57, 58, 59, 60, 61, 62, 63, and 64, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

The invention also provides an antibody, or functional fragment thereof, comprising a light chain polypeptide comprising one or more CDRs having at least one amino acid substitution in one or more light chain CDRs, the light chain CDRs selected from the group consisting of a light chain CDR1 selected from the group consisting of CDRs referenced as SEQ ID NOS:20, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, and 76; a light chain CDR2 referenced as SEQ ID NO:22:; and a light chain CDR3 selected from the group consisting of CDRs referenced as SEQ ID NOS:24, 77, 78, 79, 80, 81, 82, 83, 84, 85, and 86, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

The invention further provides an antibody, or functional fragment thereof, comprising a heavy chain polypeptide comprising one or more CDRs having at least one amino acid substitution in one or more heavy chain CDRs, the heavy chain CDRs selected from the group consisting of a heavy chain CDR1 selected from the group consisting of CDRs referenced as SEQ ID NOS:38, 87, 88, 89, 90, 91, 147 and 148; a heavy chain CDR2 selected from the group consisting of CDRs referenced as SEQ ID NOS:40, 92, 93, 94, 95 and 144; and a heavy chain CDR3 selected from the group consisting of CDRs referenced as SEQ ID NOS:42, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 and 109, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

Additionally provided is an antibody, or functional fragment thereof, comprising a light chain polypeptide comprising one or more CDRs having at least one amino acid substitution in one or more light chain CDRs, the light chain CDRs selected from the group consisting of a light chain CDR1 selected from the group consisting of CDRs referenced as SEQ ID NOS:32, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 146, 149, 150, 151, 152 and 153; a light chain CDR2 referenced as SEQ ID NOS:34, 120, 121, 122, 123, 124 and 125; and a light chain CDR3 selected from the group consisting of CDRs referenced as SEQ ID NOS:36, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 145 and 358, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope.

As described above, an antibody of the invention can be generated from any combination of the variant and/or wild type CDRs, so long as binding activity to a cryptic collagen site is maintained. As disclosed herein, a variety of combinatorial antibodies containing multiple CDRs having at least a single amino acid substitution were identified having binding activity for a cryptic collagen site. In addition to antibodies containing any combination of the respective CDRs disclosed herein, the following specific combinations of CDRs are also provided by the invention.

Exemplary HUIV26 variants include, for example, the following antibodies:

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:28; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:20; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (4.1-2D4).

An antibody comprises a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:28; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:72; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (L1b-F11).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:48; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:20; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (H2a-G8).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:154; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:157; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomA2).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:158; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomA4).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:46; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:159; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomB1).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:48; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:160; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomD2).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:72; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomD3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID

NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:157; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomD6).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:160; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomE3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:46; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:160; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomG2).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:162; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:158; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomA7).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:156; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:157; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomB10).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:26; a heavy chain CDR2 referenced as SEQ ID NO:154; a heavy chain CDR3 referenced as SEQ ID NO:63; a

light chain CDR1 referenced as SEQ ID NO:157; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomC8).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:45; a heavy chain CDR2 referenced as SEQ ID NO:155; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:157; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomD7).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:46; a heavy chain CDR2 referenced as SEQ ID NO:154; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:161; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomD11).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:46; a heavy chain CDR2 referenced as SEQ ID NO:156; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:161; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (DcomE11).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:46; a heavy chain CDR2 referenced as SEQ ID NO:28; a heavy chain CDR3 referenced as SEQ ID NO:63; a light chain CDR1 referenced as SEQ ID NO:20; a light chain CDR2 referenced as SEQ ID NO:22; and a light chain CDR3 referenced as SEQ ID NO:77 (2D4H1-C3).

Exemplary HUI77 variants include, for example, the following antibodies:

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:38; a heavy chain CDR2 referenced as SEQ ID

NO:40; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:32; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (12F10Q).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:38; a heavy chain CDR2 referenced as SEQ ID NO:92; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:32; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (QH2b-A3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:92; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:149; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1B6).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:92; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:150; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1B8).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:149; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1C3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:144; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:149; a light

chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1D3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:151; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1E3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:92; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:151; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom1H6).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:152; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:145 (Qcom1H7).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:148; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:150; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom2A4).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:115; a light

chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom2B11).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:40; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:153; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom2C1).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:92; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:116; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom2D9).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:147; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:116; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:36 (Qcom2E3).

An antibody comprising a heavy chain CDR1 referenced as SEQ ID NO:38; a heavy chain CDR2 referenced as SEQ ID NO:93; a heavy chain CDR3 referenced as SEQ ID NO:103; a light chain CDR1 referenced as SEQ ID NO:32; a light chain CDR2 referenced as SEQ ID NO:34; and a light chain CDR3 referenced as SEQ ID NO:130 (Qh2b-B7).

The invention also provides grafted antibodies containing CDRs derived from HUIV26 and HUI77, respectively. Such grafted CDRs include humanized antibodies, in which CDRs from HUIV26 or HUI77 have been grafted or in which a CDR containing one or more amino

acid substitutions is grafted. The CDRs can be grafted directly into a human framework, as disclosed herein. If desired, framework changes can also be incorporated by generating framework libraries. The optimization of CDRs and/or framework sequences can be performed independently and sequentially combined or can be performed simultaneously, as described in more detail below.

Thus, the invention additionally provides a grafted antibody in which HUIV26 CDRs (SEQ ID NOS:20, 22, 24, 26, 28 and 30) are grafted into a human framework sequence. Also provided is a grafted antibody in which HUI77 CDRs (SEQ ID NOS:32, 34, 36, 38, 40 and 42) are grafted into a human framework.

To generate grafted antibodies, donor CDRs of collagen-specific antibodies are grafted onto an antibody acceptor variable region framework. Methods for grafting antibodies and generating CDR variants to optimize activity have been described previously (WO 98/33919; WO 00/78815; WO 01/27160). The procedure can be performed to achieve grafting of donor CDRs and affinity reacquisition in a simultaneous process. The methods similarly can be used, either alone or in combination with CDR grafting, to modify or optimize the binding affinity of a variable region. The methods for conferring donor CDR binding affinity onto an acceptor variable region are applicable to both heavy and light chain variable regions and as such can be used to simultaneously graft and optimize the binding affinity of an antibody variable region.

The donor CDRs can be altered to contain a plurality of different amino acid residue changes at all or selected positions within the donor CDRs. For example, random or biased incorporation of the twenty naturally occurring amino acid residues, or preselected subsets, can be introduced into the donor CDRs to produce a diverse population of CDR species. Inclusion of CDR variant species into the diverse population of variable regions allows for the generation of variant species that exhibit optimized binding affinity for a predetermined antigen.

A range of possible changes can be made in the donor CDR positions. Some or all of the possible changes that can be selected for change can be introduced into the population of grafted donor CDRs. A single position in a CDR can be selected to introduce changes or a variety of positions having altered amino acids can be combined and screened for activity.

One approach is to change all amino acid positions along a CDR by replacement at each position with, for example, all twenty naturally occurring amino acids. The replacement of each position can occur in the context of other donor CDR amino acid positions so that a significant portion of the CDR maintains the authentic donor CDR sequence, and therefore, the binding affinity of the donor CDR. For example, an acceptor variable region framework, either a native or altered framework, can be grafted with a population of CDRs containing single position replacements at each position within the CDRs. Similarly, an acceptor variable region framework can be targeted for grafting with a population of CDRs

containing more than one position changed to incorporate all twenty amino acid residues, or a subset of amino acids. One or more amino acid positions within a CDR, or within a group of CDRs to be grafted, can be altered and grafted into an acceptor variable region framework to generate a population of grafted antibodies. It is understood that a CDR having one or more altered positions can be combined with one or more other CDRs having one or more altered positions, if desired.

A population of CDR variant species having one or more altered positions can be combined with any or all of the CDRs which constitute the binding pocket of a variable region. Therefore, an acceptor variable region framework can be targeted for the simultaneous incorporation of donor CDR variant populations at one, two or all three recipient CDR locations in a heavy or light chain. The choice of which CDR or the number of CDRs to target with amino acid position changes will depend on, for example, if a full CDR grafting into an acceptor is desired or whether the method is being performed for optimization of binding affinity.

Another approach for selecting donor CDR amino acids to change for conferring donor CDR binding affinity onto an antibody acceptor variable region framework is to select known or readily identifiable CDR positions that are highly variable. For example, the variable region CDR3 is generally highly variable. This region therefore can be selectively targeted for amino acid position changes during grafting procedures to ensure binding affinity reacquisition or augmentation, either alone or

together with relevant acceptor variable framework changes, as described herein.

If desired, CDR variant populations having one or more altered amino acid positions can be advantageously combined with a framework variant population having one or more altered amino acid positions. Such a combination can result in beneficial combinations of changes, which are identified by screening for an optimized activity.

The resultant population of CDR grafted variable regions therefore contain a species corresponding to the authentic parent amino acid residue at each position as well as a diverse number of different species which correspond to the possible combinations and permutations of the authentic parent amino acid residues together with the variant residues at each of the selected CDR positions. Such a diverse population of CDR grafted variable regions are screened for an altered variable region species which retains donor CDR binding activity, or which has optimized binding activity.

An acceptor can be selected so that it is closely similar to the variable region amino acid sequence harboring the donor CDRs. In addition, a variety of acceptors less closely related to the donor antibody can be used. Alternatively, a library of all possible or relevant changes in the acceptor framework can be made and then screened for those variable regions, or heteromeric binding fragments thereof, that maintain or exhibit increased binding affinity compared to the donor molecule. The donor CDRs can be grafted into a

variety of naturally occurring acceptor frameworks or altered frameworks having one or more changes or even a library containing changes at one or more positions. Therefore, the applicability is not preconditioned on the availability or search for an acceptor framework variable region similar to that of the donor.

The methods for conferring donor CDR binding affinity onto a variable region can involve identifying the relevant amino acid positions in the acceptor framework that are known or predicted to influence a CDR conformation, or that are known or predicted to influence the spacial context of amino acid side chains within the CDR that participate in binding, and then generating a population of altered variable region species that incorporate a plurality of different amino acid residues at those positions. For example, the different amino acid residues at those positions can be incorporated either randomly or with a predetermined bias and can include all of the twenty naturally occurring amino acid residues at each of the relevant positions. Subsets, including less than all of the naturally occurring amino acids can additionally be chosen for incorporation at the relevant framework positions. Including a plurality of different amino acid residues at each of the relevant framework positions ensures that there will be at least one species within the population that will have framework changes which allows the CDRs to reacquire their donor binding affinity in the context of the acceptor framework variable region.

For humanizing an antibody, any of a variety of human frameworks can be selected for CDR grafting. For

example, CDRs of HUIV26 or HUI77 can be cloned into a variety of human framework sequences. The frameworks can be generated using human germline genes encoding heavy and light chain variable regions as well as J regions to obtain human framework sequences for CDR grafting. Exemplary human framework nucleotide sequences include, for example, the framework sequences of DPK24 (VKIV) (SEQ ID NO:5), DP-54 (VHIII) (SEQ ID NO:7), DPK13 (VKII) (SEQ ID NO:13), DP-28 (VHII) (SEQ ID NO:15), as well as J regions JK1 (SEQ ID NO:217), JK2 (SEQ ID NO:218) and JH6 (SEQ ID NO:219). It is understood that framework regions from any available germline sequence can be combined with any available J sequence, as desired, to generate a human framework for grafting CDRs. For example, an alignment of mouse variable regions of HUIV26 and HUI77 with an exemplary human framework is shown in Figures 2C and 3C, respectively. A fusion of VKIV/JK2 light chain variable region and VHIII/JH6 heavy chain variable region are aligned with HUIV26 (Figure 2C). A fusion of VKII/JK1 light chain variable region and VHIII/JH6 heavy chain variable region are aligned with HUI77 (Figure 3C). An exemplary fusion of a germline and J region is shown in Figure 3D, which is aligned with the HUI77 light chain. It is understood that any available human framework can be selected for CDR grafting and, if desired, optimized by the methods disclosed herein. As disclosed herein, CDRs having beneficial mutations can be grafted into a variety of frameworks and have retained or improved activity (see Example III).

Selection of the relevant framework amino acid positions to alter depends on a variety of criteria well known to those skilled it the art. One criteria for

selecting relevant framework amino acids to change can be the relative differences in amino acid framework residues between the donor and acceptor molecules. Selection of relevant framework positions to alter using this approach is simple and has the advantage of avoiding any subjective bias in residue determination or any bias in CDR binding affinity contribution by the residue.

Another criteria that can be used for determining the relevant amino acid positions to change can be, for example, selection of framework residues that are known to be important or to contribute to CDR conformation. For example, canonical framework residues are important for CDR conformation or structure. Targeting of a canonical framework residue as a relevant position to change can identify a more compatible amino acid residue in context with its associated donor CDR sequence.

The frequency of an amino acid residue at a particular framework position is another criteria which can be used for selecting relevant framework amino acid positions to change. For example, comparison of the selected framework with other framework sequences within its subfamily can reveal residues that occur at minor frequences at a particular position or positions. Such positions harboring less abundant residues are similarly applicable for selection as a position to alter in the acceptor variable region framework.

The relevant amino acid positions to change also can be selected, for example, based on proximity to a CDR. In certain contexts, such residues can

participate in CDR conformation or antigen binding. Moreover, this criteria can similarly be used to prioritize relevant positions selected by other criteria described herein. Therefore, differentiating between residues proximal and distal to one or more CDRs is an efficient way to reduce the number of relevant positions to change.

Other criteria for selecting relevant amino acid framework positions to alter include, for example, residues that are known or predicted to reside in three-dimensional space near the antigen-CDR interface or predicted to modulate CDR activity. Similarly, framework residues that are known or predicted to form contacts between the heavy (V_H) and light (V_L) chain variable region interface can be selected. Such framework positions can affect the conformation or affinity of a CDR by modulating the CDR binding pocket, antigen interaction or the V_H and V_L interaction. Therefore, selection of these amino acid positions for constructing a diverse population for screening of binding activity can be used to identify framework changes which replace residues having detrimental effects on CDR conformation or compensate for detrimental effects of residues occurring elsewhere in the framework.

Other framework residues that can be selected for alteration include amino acid positions that are inaccessible to solvent. Such residues are generally buried in the variable region and are therefore capable of influencing the conformation of the CDR or V_H and V_L interactions. Solvent accessibility can be predicted, for example, from the relative hydrophobicity of the

environment created by the amino acid side chains of the polypeptide or by known three-dimensional structural data.

Following selection of relevant amino acid positions in the donor CDRs, as well as any relevant amino acid positions in the framework regions desired to be varied, amino acid changes at some or all of the selected positions can be incorporated into encoding nucleic acids for the acceptor variable region framework and donor CDRs. Altered framework or CDR sequences can be individually made and tested, or can be simultaneously combined and tested, if desired.

The variability at any or all of the altered positions can range from a few to a plurality of different amino acid residues, including all twenty naturally occurring amino acids or functional equivalents and analogues thereof.

Selection of the number and location of the amino acid positions to vary is flexible and can depend on the intended use and desired efficiency for identification of the altered variable region having a desirable activity such as substantially the same or greater binding affinity compared to the donor variable region. In this regard, the greater the number of changes that are incorporated into a altered variable region population, the more efficient it is to identify at least one species that exhibits a desirable activity, for example, substantially the same or greater binding affinity as the donor. Alternatively, where the user has empirical or actual data to the affect that certain amino

acid residues or positions contribute disproportionally to binding affinity, then it can be desirable to produce a limited population of altered variable regions which focuses on changes within or around those identified residues or positions.

For example, if CDR grafted variable regions are desired, a large, diverse population of altered variable regions can include all the non-identical framework region positions between the donor and acceptor framework and all single CDR amino acid position changes. Alternatively, a population of intermediate diversity can include subsets, for example, of only the proximal non-identical framework positions to be incorporated together with all single CDR amino acid position changes. The diversity of the above populations can be further increased by, for example, additionally including all pairwise CDR amino acid position changes. In contrast, populations focusing on predetermined residues or positions which incorporate variant residues at as few as one framework and/or one CDR amino acid position can similarly be constructed for screening and identification of an altered antibody variable region of the invention. As with the above populations, the diversity of such focused populations can be further increased by additionally expanding the positions selected for change to include other relevant positions in either or both of the framework and CDR regions. There are numerous other combinations ranging from few changes to many changes in either or both of the framework regions and CDRs that can additionally be employed, all of which will result in a population of altered variable regions that can be screened for the identification of at least one CDR

grafted altered variable region having desired activity, for example, binding activity to a cryptic collagen site. Those skilled in the art will know, or can determine, which selected residue positions in the framework or donor CDRs, or subsets thereof, can be varied to produce a population for screening and identification of an altered antibody of the invention given the teachings and guidance provided herein.

Simultaneous incorporation of all of the CDR encoding nucleic acids and all of the selected amino acid position changes can be accomplished by a variety of methods known to those skilled in the art, including for example, recombinant and chemical synthesis. For example, simultaneous incorporation can be accomplished by, for example, chemically synthesizing the nucleotide sequence for the acceptor variable region, fused together with the donor CDR encoding nucleic acids, and incorporating at the positions selected for harboring variable amino acid residues a plurality of corresponding amino acid codons.

One such method well known in the art for rapidly and efficiently producing a large number of alterations in a known amino acid sequence or for generating a diverse population of variable or random sequences is known as codon-based synthesis or mutagenesis. This method is the subject matter of U.S. Patent Nos. 5,264,563 and 5,523,388 and is also described in Glaser et al. J. Immunology 149:3903 (1992). Briefly, coupling reactions for the randomization of, for example, all twenty codons which specify the amino acids of the genetic code are performed in separate reaction vessels

and randomization for a particular codon position occurs by mixing the products of each of the reaction vessels. Following mixing, the randomized reaction products corresponding to codons encoding an equal mixture of all twenty amino acids are then divided into separate reaction vessels for the synthesis of each randomized codon at the next position. For the synthesis of equal frequencies of all twenty amino acids, up to two codons can be synthesized in each reaction vessel.

Variations to these synthesis methods also exist and include for example, the synthesis of predetermined codons at desired positions and the biased synthesis of a predetermined sequence at one or more codon positions. Biased synthesis involves the use of two reaction vessels where the predetermined or parent codon is synthesized in one vessel and the random codon sequence is synthesized in the second vessel. The second vessel can be divided into multiple reaction vessels such as that described above for the synthesis of codons specifying totally random amino acids at a particular position. Alternatively, a population of degenerate codons can be synthesized in the second reaction vessel such as through the coupling of NNG/T nucleotides where N is a mixture of all four nucleotides. Following synthesis of the predetermined and random codons, the reaction products in each of the two reaction vessels are mixed and then redivided into an additional two vessels for synthesis at the next codon position.

A modification to the above-described codon-based synthesis for producing a diverse number of variant sequences can similarly be employed for the

production of the variant populations described herein. This modification is based on the two vessel method described above, which biases synthesis toward the parent sequence and allows the user to separate the variants into populations containing a specified number of codon positions that have random codon changes.

Briefly, this synthesis is performed by continuing to divide the reaction vessels after the synthesis of each codon position into two new vessels. After the division, the reaction products from each consecutive pair of reaction vessels, starting with the second vessel, is mixed. This mixing brings together the reaction products having the same number of codon positions with random changes. Synthesis proceeds by then dividing the products of the first and last vessel and the newly mixed products from each consecutive pair of reaction vessels and redividing into two new vessels. In one of the new vessels, the parent codon is synthesized and in the second vessel, the random codon is synthesized. For example, synthesis at the first codon position entails synthesis of the parent codon in one reaction vessel and synthesis of a random codon in the second reaction vessel. For synthesis at the second codon position, each of the first two reaction vessels is divided into two vessels yielding two pairs of vessels. For each pair, a parent codon is synthesized in one of the vessels and a random codon is synthesized in the second vessel. When arranged linearly, the reaction products in the second and third vessels are mixed to bring together those products having random codon sequences at single codon positions. This mixing also reduces the product populations to three, which are the

starting populations for the next round of synthesis. Similarly, for the third, fourth and each remaining position, each reaction product population for the preceding position are divided and a parent and random codon synthesized.

Following the above modification of codon-based synthesis, populations containing random codon changes at one, two, three and four positions as well as others can be conveniently separated out and used based on the need of the individual. Moreover, this synthesis scheme also allows enrichment of the populations for the randomized sequences over the parent sequence since the vessel containing only the parent sequence synthesis is similarly separated out from the random codon synthesis.

Other methods well known in the art for producing a large number of alterations in a known amino acid sequence or for generating a diverse population of variable or random sequences include, for example, degenerate or partially degenerate oligonucleotide synthesis. Codons specifying equal mixtures of all four nucleotide monomers, represented as NNN, results in degenerate synthesis. Whereas partially degenerate synthesis can be accomplished using, for example, the NNG/T codon described previously. Other methods well known in the art can alternatively be used such as the use of statistically predetermined, or varigated, codon synthesis, which is the subject matter of U.S. Patent Nos. 5,223,409 and 5,403,484.

Once the populations of altered variable region encoding nucleic acids have been constructed as described

above, they can be expressed to generate a population of altered variable region polypeptides that can be screened for binding affinity. For example, the altered variable region encoding nucleic acids can be cloned into an appropriate vector for propagation, manipulation and expression. Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements sufficient for the transcription, translation, regulation, and if desired, sorting and secretion of the altered variable region polypeptides. The vectors can be suitable for expression in either procaryotic or eukaryotic host systems so long as the expression and regulatory elements function in the respective host system. The expression vectors can additionally include regulatory elements for inducible or cell type-specific expression. One skilled in the art will know which host systems are compatible with a particular vector and which regulatory or functional elements are sufficient to achieve expression of the polypeptides in soluble, secreted or cell surface forms.

Appropriate host cells, include for example, bacteria and corresponding bacteriophage expression systems, yeast, avian, insect and mammalian cells. Methods for recombinant expression, screening and purification of populations of altered variable regions or altered variable region polypeptides within such populations in various host systems are well known in the art and are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992) and in Ausubel et al., Current Protocols in Molecular Biology, (Supplement 54), John Wiley & Sons, New York (2001). The choice of a

particular vector and host system for expression and screening of altered variable regions are known to those skilled in the art and will depend on the preference of the user. Moreover, expression of diverse populations of hetereomeric receptors in either soluble or cell surface form using filamentous bacteriophage vector/host systems is well known in the art and is the subject matter of U.S. Patent No. 5,871,974.

The expressed population of altered variable region polypeptides can be screened for the identification of one or more altered variable region species exhibiting optimized binding activity, for example, binding affinity substantially the same or greater than the donor CDR variable region. Screening can be accomplished using various methods well known in the art for determining the binding affinity of a polypeptide or compound. Additionaly, methods based on determining the relative affinity of binding molecules to their partner by comparing the amount of binding between the altered variable region polypeptides and the donor CDR variable region can similarly be used for the identification of species exhibiting binding affinity substantially the same or greater than the donor CDR variable region. All of such methods can be performed, for example, in solution or in solid phase. Moreover, various formats of binding assays are well known in the art and include, for example, immobilization to filters such as nylon or nitrocellulose; two-dimensional arrays, enzyme linked immunosorbant assay (ELISA), radioimmunoassay (RIA), panning and plasmon resonance. Such methods can be found described in, for example, Harlow and Lane, *supra*, 1988.

For the screening of populations of polypeptides such as the altered variable region populations produced by the methods of the invention, immobilization of the populations of altered variable regions to filters or other solid substrate can be advantageous because large numbers of different species can be efficiently screened for antigen binding. Such filter lifts allow for the identification of altered variable regions that exhibit substantially the same or greater binding affinity compared to the donor CDR variable region. Alternatively, if the populations of altered variable regions are expressed on the surface of a cell or bacteriophage, panning on immobilized antigen can be used to efficiently screen for variants having antigen binding activity or to determine the relative binding affinity of species within the population.

Another affinity method for screening populations of altered variable regions polypeptides is a capture lift assay that is useful for identifying a binding molecule having selective affinity for a ligand (Watkins et. al., (1997); WO 99/06834). This method employs the selective immobilization of altered variable regions to a solid support and then screening of the selectively immobilized altered variable regions for selective binding interactions against the cognate antigen or binding partner. Selective immobilization functions to increase the sensitivity of the binding interaction being measured since initial immobilization of a population of altered variable regions onto a solid support reduces non-specific binding interactions with irrelevant molecules or contaminants which can be present in the reaction.

Another method for screening populations or for measuring the affinity of individual altered variable region polypeptides is through surface plasmon resonance (SPR). This method is based on the phenomenon which occurs when surface plasmon waves are excited at a metal/liquid interface. Light is directed at, and reflected from, the side of the surface not in contact with sample, and SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. Biomolecular binding events cause changes in the refractive index at the surface layer, which are detected as changes in the SPR signal. The binding event can be either binding association or disassociation between a receptor-ligand pair. The changes in refractive index can be measured essentially instantaneously and therefore allows for determination of the individual components of an affinity constant. More specifically, the method enables accurate measurements of association rates (k_{on}) and disassociation rates (k_{off}).

Measurements of k_{on} and k_{off} values can be used identify altered variable regions or optimized variable regions that are therapeutically more efficacious. For example, an altered variable region, or heteromeric binding fragment thereof, can be more efficacious because it has, for example, a higher k_{on} valued compared to variable regions and heteromeric binding fragments that exhibit similar binding affinity. Increased efficacy is conferred because molecules with higher k_{on} values can specifically bind and inhibit their target at a faster rate. Similarly, a molecule of the invention can be more efficacious because it exhibits a lower k_{off} value compared to molecules having similar binding affinity.

Increased efficacy observed with molecules having lower k_{off} rates can be observed because, once bound, the molecules are slower to dissociate from their target. Although described with reference to the altered variable regions and optimized variable regions of the invention, the methods described above for measuring association and dissociation rates are applicable to essentially any antibody or fragment thereof for identifying more effective binders for therapeutic or diagnostic purposes.

Methods for measuring the affinity, including association and dissociation rates using surface plasmon resonance are well known in the art and can be found described in, for example, Jönsson and Malmquist, Advances in Biosensors, 2:291-336 (1992) and Wu et al. Proc. Natl. Acad. Sci. USA, 95:6037-6042 (1998). Moreover, one apparatus well known in the art for measuring binding interactions is a BIAcore 2000 instrument which is commercially available through Pharmacia Biosensor, (Uppsala, Sweden).

Using any of the above described screening methods, as well as others well known in the art, an altered variable region having optimized binding activity, for example, binding affinity substantially the same or greater than the donor CDR variable region is identified by detecting the binding of at least one altered variable region within the population to its antigen or cognate ligand. In addition to optimizing for antigen binding activity, catalytic activity can also be included in an invention antibody and optimized using the methods disclosed herein for binding affinity optimization. Accordingly, the above methods can be

modified to include the addition of substrate and reactants to screen for optimized catalytic activity. Comparison, either independently or simultaneously in the same screen, with the donor variable region will identify those binders that have substantially the same or greater binding affinity as the donor. Those skilled in the art will know, or can determine using the donor variable region, binding conditions which are sufficient to identify selective interactions over non-specific binding.

Detection methods for identification of binding species within the population of altered variable regions can be direct or indirect and can include, for example, the measurement of light emission, radioisotopes, colorimetric dyes and fluorochromes. Direct detection includes methods that function without intermediates or secondary measuring procedures to assess the amount of bound antigen or ligand. Such methods generally employ ligands that are themselves labeled with a detectable moiety, for example, a radioactive, light emitting, fluorescent, colorimetric or enzyme moiety. In contrast, indirect detection includes methods that function through an intermediate or secondary measuring procedure. These methods generally employ molecules that specifically react with the antigen or ligand and can themselves be directly labeled with a detectable moiety or detected by a secondary reagent. For example, an antibody specific for a ligand can be detected using a secondary antibody capable of interacting with the first antibody specific for the ligand, again using the detection methods described above for direct detection. Moreover, for the specific example of screening for catalytic antibodies,

the disappearance of a substrate or the appearance of a product can be used as an indirect measure of binding affinity or catalytic activity.

Isolated variable regions exhibit binding affinity as single chains, in the absence of assembly into a heteromeric structure with their respective V_H or V_L subunits. As such, populations of V_H and V_L altered variable regions polypeptides can be expressed alone and screened for binding activity, for example, optimized activity having substantially the same or greater binding affinity compared to the CDR donor V_H or V_L variable region. Alternatively, populations of V_H and V_L altered variable regions polypeptides can be coexpressed so that they self-assemble into heteromeric altered variable region binding fragments. The heteromeric binding fragment population can then be screened for species exhibiting binding affinity substantially the same or greater than the CDR donor variable region binding fragment.

Employing the methods for simultaneously grafting and optimizing, or for optimizing, it is possible to generate heteromeric variable region binding fragments having increases in affinities of greater than about 2-fold, 3-fold, 4-fold, 5-fold, 8-fold or 10-fold. In particular, heteromeric variable region binding fragments can be generated having increases in affinities of greater than 12-fold, 15-fold, 20-fold, and 25-fold as well as affinities greater than 50-fold, 100-fold, 200-fold, 500-fold or 1000-fold compared to the donor or parent molecule.

Additionally, the methods described herein for optimizing are also are applicable for producing catalytic heteromeric variable region fragments or for optimizing their catalytic activity. Catalytic activity can be optimized by changing, for example, the on or off rate of substrate binding, the substrate binding affinity, the transition state binding affinity, the turnover rate (kcat) or the Km. Methods for measuring these characteristics are well known in the art (see, for example Segel, Enzyme Kinetics, John Wiley & Sons, New York (1975)). Such methods can be employed in the screening steps of the methods described above when used for optimizing the catalytic activity of a heteromeric variable region binding fragment.

Additionally, the methods for conferring donor CDR binding affinity onto an antibody acceptor variable region framework are applicable for grafting CDRs as described by Kabat et al., *supra*, Chothia et al., *supra* or MacCallum et al., *supra*. The methods similarly can be used for grafting into an acceptor framework overlapping regions or combinations of CDRs as described in Kabat et al., *supra*, Chothia et al., *supra* or MacCallum et al., *supra*. Generally, variable region CDRs are grafted by identifying the boundries described by one of the CDR definitions known in the art and set forth herein. However, because the methods are directed to constructing and screening populations of CDR grafted altered variable regions, which can incorporate relevant amino acid position changes in both the framework and CDR regions, and such variations can, for example, compensate or augment amino acid changes elsewhere in the variable region, the exact boundry of a particular CDR or set of

variable region CDRs can be varied. Therefore, the exact CDR region to graft, whether it is the region described by Kabat et al., Chothia et al. or MacCallum et al., or any combination thereof, will essentially depend on the preference of the user.

Similarly, the methods described previously for optimizing the binding affinity of an antibody also are applicable for use with essentially any variable region for which an encoding nucleic acid is, or can be made, available. As with the methods for conferring donor CDR binding affinity, many applications of the methods for optimizing binding affinity will be for modifying the binding affinity of CDR grafted variable regions having human frameworks. Again, such molecules are significantly less antigenic in human patients and therefore therapeutically valuable in the treatment of human diseases. However, the methods of the invention for optimizing the binding affinity of a variable region are applicable to all species of variable regions. Therefore, the invention includes binding affinity optimization of variable regions derived from human, mouse, rat, rabbit, goat and chicken, or any other desired species.

The methods of the invention have been described with reference to variable regions and heteromeric variable region binding fragments. Those skilled in the art will understand that all of such methods are applicable to whole antibodies and functional fragments thereof as well as to regions and functional domains other than the antigen binding variable region of antibodies, if desired.

An association rate can be determined in any non-equilibrium mixture including, for example, one formed by rapidly contacting a binding polypeptide and ligand or by rapidly changing temperature. A non-equilibrium mixture can be a pre-equilibrium mixture. A pre-equilibrium mixture can be formed, for example, by contacting a soluble binding polypeptide and soluble ligand in a condition where the amount of total ligand and total binding polypeptide in the detection chamber are constant. Measurements of association rates in pre-equilibrium mixtures can be made in formats providing rapid mixing of binding polypeptide with ligand and rapid detection of changing properties of the binding polypeptide or ligand on a timescale of milliseconds or faster. Stopped flow and rapid quench flow instruments such as those described below provide a convenient means to measure non-equilibrium kinetics. The association rate can also be measured in non-equilibrium mixtures including, for example, solutions containing insoluble species of binding polypeptide, ligand or binding polypeptide bound to ligand, or solutions containing variable concentrations of total ligand or total binding polypeptide. Measurement of an association rate in a non-equilibrium mixture can be made in formats providing attachment of a ligand to a surface and continuous flow of a solution containing the binding polypeptide over the surface, or vice-versa, combined with rapid detection of changing properties of the binding polypeptide, ligand or surface such that measurements are made on a timescale of milliseconds or faster. Examples of formats providing non-equilibrium measurement of association rates include surface plasmon resonance instruments and evanescent wave instruments.

Association rate measurements can be made by detecting the change in a property of the binding polypeptide or ligand that exists between the bound and unbound state or by detecting a change in the surrounding environment when binding polypeptide and ligand associate. Properties of the binding polypeptide or ligand that can change upon association and that can be used to measure association rates include, for example, absorption and emission of heat, absorption and emission of electromagnetic radiation, affinity for a receptor, molecular weight, density, mass, electric charge, conductivity, magnetic moment of nuclei, spin state of electrons, polarity, molecular shape, or molecular size. Properties of the surrounding environment that can change when binding polypeptide associates with ligand include, for example, temperature and refractive index of surrounding solvent.

Formats for measuring association rates in pre-equilibrium mixtures include, for example, stopped flow kinetic instruments and rapid quench flow instruments. A stopped flow instrument can be used to push solutions containing a binding polypeptide and ligand from separate reservoirs into a mixing chamber just prior to passage into a detection cell. The instrument can then detect a change in one or more of the above described properties to monitor progress of the binding event. A rapid quench flow instrument can be used to rapidly mix a solution containing a binding polypeptide with a solution containing a ligand followed by quenching the binding reaction after a finite amount of time. A change in one or more of the above described properties can then be detected for quenched mixtures produced by quenching at

different times following mixing. Quenching can be performed for example by freezing or addition of a chemical quenching agent so long as the quenching step does not inhibit detection of the property relied upon for measurement of binding rate. Thus, a rapid quench instrument can be useful, for example, in situations where spectroscopic detection is not convenient. A variety of instruments are commercially available from vendors such as KinTek Corp. (State College, PA) and Hi-Tech Scientific (Salisbury, UK).

Formats for measuring association rates in non-equilibrium mixtures include, for example, surface plasmon resonance and evanescent wave instruments. Surface plasmon resonance and evanescent wave technology utilize a ligand or binding polypeptide attached to a biosensor surface and a solution containing either the binding polypeptide or ligand respectively that is passed over the biosensor surface. The change in refractive index of the solution that occurs at the surface of a chip when binding polypeptide associates with ligand can be measured in a time dependent fashion. For example, surface plasmon resonance is based on the phenomenon which occurs when surface plasmon waves are excited at a metal/liquid interface. Light is directed at, and reflected from, the side of the surface not in contact with sample, and SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. Biomolecular binding events cause changes in the refractive index at the surface layer, which are detected as changes in the SPR signal. The binding event can be either binding association or disassociation between a receptor-ligand pair. The changes in

refractive index can be measured essentially instantaneously and therefore allows for determination of the individual components of an affinity constant. More specifically, the method enables accurate measurements of association rates (k_{on}) and disassociation rates (k_{off}). Surface plasmon resonance instruments are available in the art including, for example, the BIAcore instrument, IBIS system, SPR-CELLIA system, Spreeta, and Plasmon SPR and evanescent wave technology is available in the Iasys system as described, for example, in Rich and Myszka, Curr. Opin. Biotech. 11:54-61 (2000).

Another method for measuring binding affinity includes comparative ELISA. As disclosed herein, an approximation of changes in affinity based on shifts in half-maximal binding was used to identify k_{on} and k_{off} values relative to wild type (Example III). Such a method is particularly useful for screening large numbers of variants, whereas the above-described methods can be used for detailed analysis of binding activity.

The association rate can be determined by measuring a change in a property of a ligand or binding polypeptide at one or more discreet time intervals during the binding event using, for example, the methods described above. Measurements determined at discreet time intervals during the binding event can be used to determine a quantitative measure of association rate or a relative measure of association rate. Quantitative measures of association rate can include, for example, an association rate value or k_{on} value. Quantitative values of association rate or k_{on} can be determined from a mathematical or graphical analysis of a time dependent

measurement. Such analyses are well known in the art and include algorithms for fitting data to a sum of exponential or linear terms or algorithms for computer simulation to fit data to a binding model as described for example in Johnson, Cur. Opin. Biotech. 9:87-89 (1998), which is incorporated herein by reference.

Association rates can be determined from mixtures containing insoluble species or variable concentrations of total ligand or total binding polypeptide using mathematical and graphical analyses such as those described above if effects of mass transport are accounted for in the reaction. One skilled in the art can account for mass transport by comparing association rates under conditions having similar limitations with respect to mass transport or by adjusting the calculated association rate according to models available in the art including, for example those described in Myszka et al., Biophys. J. 75:583-594 (1998), which is incorporated herein by reference.

A higher value of either the association rate or k_{on} is generally indicative of improved therapeutic potency. Thus, quantitative determinations provide an advantage by allowing comparison between an association rate of a binding polypeptide and a therapeutic control determined by different methods so long as the methods used are understood by one skilled in the art to yield consistent results.

A relative measure of association rate can include, for example, comparison of association rate for two or more binding polypeptides binding to ligand under

similar conditions or comparison of association rate for a binding polypeptide binding to ligand with a predefined rate. Comparison of association rate for two or more binding polypeptides can include a standard of known association rate or a molecule of known therapeutic effect. A predefined rate used for comparison can be determined by calibrating the measurement relative to a previously measured rate including, for example, one available in the scientific literature or in a database. An example of a comparison with a predefined rate is selection of the species of binding polypeptide bound to ligand at a discreet time interval defined by the predefined rate by using a time actuated selection device.

For purposes of comparison, the association rate of a binding polypeptide and ligand can be determined relative to association rate for a therapeutic control and the same ligand. A comparison can also be made according to a quantitative association rate for binding polypeptide and ligand compared to a quantitative association rate for a therapeutic control and ligand. Relative or quantitative association rates can be determined by the methods described above. Determination of association rates for a binding polypeptide associating with a ligand can be performed simultaneously with a binding polypeptide and therapeutic control or at separate times, provided conditions are sufficiently similar in each assay to allow valid comparison. Thus, association rate determined for a binding polypeptide can be compared to a previously measured association rate for a therapeutic control.

A binding polypeptide having improved therapeutic potency can be distinguished from a binding polypeptide that has an increased K_a for a ligand but not improved therapeutic potency. Methods for identifying a therapeutic binding polypeptide based on K_a rely on an equilibrium measurement which, absent time dependent measurements made in a non-equilibrium condition, are inaccurate for identifying a binding polypeptide having increased association rate and therefore improved therapeutic potency. According to the relationship $K_a = k_{on}/k_{off}$, an increased K_a for association of a binding polypeptide and ligand can be due to changes in k_{on} or k_{off}. For example, a binding polypeptide having improved therapeutic potency can have a reduced K_a if a reduction in k_{off} occurs that over compensates for an increase in k_{on}. Thus, changes in K_a, being influenced by changes in k_{off}, do not unambiguously correlate with changes in therapeutic potency since binding polypeptides having improved therapeutic potency can display either reduced or increased K_a.

For optimization of binding activity of an antibody of the invention, the fold increase in association rate can be indicated by an increase in k_{on}. Therefore, k_{on} can be about 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold or more using methods described herein. The k_{on} can be at least about 1×10^2 $M^{-1}s^{-1}$, 2×10^2 $M^{-1}s^{-1}$, 5×10^2 $M^{-1}s^{-1}$, 1×10^3 $M^{-1}s^{-1}$, 2×10^3 $M^{-1}s^{-1}$, 5×10^3 $M^{-1}s^{-1}$, 1×10^4 $M^{-1}s^{-1}$, 2×10^4 $M^{-1}s^{-1}$, 5×10^4 $M^{-1}s^{-1}$, 1×10^5 $M^{-1}s^{-1}$, 2×10^5 $M^{-1}s^{-1}$, or 3×10^5 $M^{-1}s^{-1}$. The k_{on} can also be increased to at least about 5×10^5 $M^{-1}s^{-1}$, 7×10^5 $M^{-1}s^{-1}$, 9×10^5 $M^{-1}s^{-1}$, 1×10^6 $M^{-1}s^{-1}$, 3×10^6 $M^{-1}s^{-1}$, 5×10^6 $M^{-1}s^{-1}$, 7×10^6 $M^{-1}s^{-1}$, 9×10^6 $M^{-1}s^{-1}$ or $1 \times$

10^7 $M^{-1}s^{-1}$ or more. Furthermore, the increase in k_{on} resulting in improved therapeutic potency can be independent of an effect of a change in K_a for the binding polypeptide. The binding polypeptide having an increase in k_{on} can have a K_a value similar to K_a for its parent polypeptide or a K_a value lower than K_a for its parent polypeptide.

The invention also provides nucleic acids encoding the antibodies and CDRs of the invention. The invention further provides nucleic acids encoding the mouse antibodies HUIV26 (SEQ ID NOS:1 and 3) and HUI77 (SEQ ID NOS:5 and 7) (see Figures 2 and 3). Further provided are nucleic acids encoding HUIV26 CDRs (SEQ ID NOS:20, 22, 24, 26, 28 and 30) and encoding HUI77 CDRs (SEQ ID NOS:32, 34, 36, 38, 40 and 42). Such nucleic acids include nucleic acids having degenerate codons encoding any or all of the amino acids in the CDRs. For example, the invention provides nucleic acids encoding HUIV26 CDRs: V_L CDR1, SEQ ID NOS:19; V_L CDR2, SEQ ID NO:21; V_L CDR3, SEQ ID NO:23; V_H CDR1, SEQ ID NO:25; V_H CDR2, SEQ ID NO:27; and V_H CDR3, SEQ ID NO:29. The invention also provides nucleic acids encoding HUI77 CDRs: V_L CDR1, SEQ ID NOS:31; V_L CDR2, SEQ ID NO:33; V_L CDR3, SEQ ID NO:35; V_H CDR1, SEQ ID NO:37; V_H CDR2, SEQ ID NO:39; and V_H CDR3, SEQ ID NO:41. Also included are degenerate versions of such nucleic acids such that they encode the amino acid sequences referenced as SEQ ID NOS:20, 22, 24, 26, 28 and 30 for HUIV26 and SEQ ID NOS:32, 34, 36, 38, 40 and 42 for HUI77.

Further provided are nucleic acids encoding a HUIV26 or HUI77 CDR containing one or more amino acid

subsitutions. For example, the invention provides nucleic acids encoding the CDRs of HUIV26 and HUI77 having single or multiple amino acid substitutions, as disclosed herein. If a nucleic acid encoding a CDR having one or more amino acid substitution is derived, for example, from one of SEQ ID NOS:19, 21, 23, 25, 27 or 29 for HUIV26 or SEQ ID NOS:31, 33, 35, 37, 39 or 41 for HUI77, the amino acid substitutions can be encoded by any of the corresponding degenerate codons for that amino acid. Nucleic acids encoding such CDR variants can also include degenerate codons at any or all of the wild type amino acid positions.

Throughout the application, various nucleic acids and oligonucleotide primers, in addition to the naturally occurring nucleotides A, C, G, T or U, refer to standard abbreviations: R = G or A; Y = T/U or C; M = A or C; K = G or T/U; S = G or C; W = A or T/U; B = G, C or T/U; D = A, G or T/U; H = A, C or T/U; V = A, G or C; N = any nucleotide.

The antibodies of the invention have binding activity for a cryptic collagen epitope. The HUIV26 and HUI77 antibodies have been shown to target to angiogenic vasculature (see Xu et al., *supra*, 2001; WO 00/40597). Accordingly, the grafted HUIV26 and HUI77 antibodies of the invention, which specifically bind to a cryptic collagen epitope, similarly can target to angiogenic vasculature. One of the most significant and important aspects of the monoclonal antibodies HUIV26 and HUI77, and the grafted forms thereof disclosed herein, is that of their specificity. It is expected that systemic administration of antibodies of the invention will have

minimal if any toxic side effects since the cryptic epitope(s) that is recognized by the HUIV26 and HUI77 antibodies is/are not exposed in mature native triple helical collagen but is only exposed upon denaturaion, for example, heat denaturation or proteolytic denaturation. Thus, little, if any, binding under normal physiological conditions is expected.

Moreover, the cryptic collagen domain(s) to which HUIV26 and HUI77 bind represents a novel therapeutic target for the treatment of numerous neovascular diseases including tumor growth and metastasis, diabetic retinopathy and other related ocular diseases such as macular degeneration, psoriasis, and rheumatoid arthritis. Other exemplary diseases associated with angiogenesis include, but are not limited to, inflammatory disorders such as immune and non-immune inflammation, chronic articular rheumatism and psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma, restenosis, capillary proliferation in atherosclerotic plaques and osteoporosis, and cancer associated disorders, such as solid tumors, solid tumor metastases, angiofibromas, retrolental fibroplasia, hemangiomas, Kaposi's sarcoma and the like cancers which require neovascularization to support tumor growth. Other exemplary tumors include melanoma, carcinoma, sarcoma, fibrosarcoma, glioma and astrocytoma, and the like.

Thus, the methods of the invention can be used to treat an individual having a disease associated with angiogenesis, including those described above. The

methods can be used to ameliorate a sign or symptom associated with a disease. For example, in the case of cancer treatment, the methods can be used to inhibit tumor growth. One skilled in the art will know or can readily determine an appropriate sign or symptom associated with a disease suitable for determining the effectiveness of a therapeutic application using an antibody of the invention.

The antibodies of the invention can also be used as an important diagnostic and imaging reagent for the early detection of aberrant neovascularization associated with invasive tumor growth and metastasis. The antibodies of the invention can also be used in staging and grading of tumors since invasive tumor in contrast to benign lesions are likely to be associated with degradation of the surrounding basement membrane.

Thus, the invention provides a method of targeting angiogenic vasculature, comprising administering an antibody, or functional fragment thereof, the antibody or functional fragment thereof having specific binding activity for a cryptic collagen epitope, wherein the antibody or functional fragment is an antibody of the invention. For example, the antibodies can comprise one or more CDRs, including wild type CDRs or variants thereof, of the HUIV26 and HUI77 antibodies, as disclosed herein. The methods of targeting angiogenic vasculature can be used for therapeutic and/or diagnostic purposes.

For therapeutic purposes, the antibody, or functional fragment thereof, can be administered as a

therapeutic agent itself or can further comprise a therapeutic moiety. In the case of a therapeutic moiety, the moiety can be a drug such as a chemotherapeutic agent, cytotoxic agent, toxin, or anti-angiogenic agent, which refers to a molecule that reduces or inhibits angiogenesis. For example, a cytotoxic agent can be a radionuclide or chemical compound. Exemplary radionuclides useful as therapeutic agents include, for example, X-ray or γ-ray emitters. In addition, a moiety can be a drug delivery vehicle such as a chambered microdevice, a cell, a liposome or a virus, which can contain an agent such as a drug or a nucleic acid.

Exemplary therapeutic agents include, for example, the anthracyclin, doxorubicin, which has been linked to antibodies and the antibody/doxorubicin conjugates have been therapeutically effective in treating tumors (Sivam et al., Cancer Res. 55:2352-2356 (1995); Lau et al., Bioorg. Med. Chem. 3:1299-1304 (1995); Shih et al., Cancer Immunol. Immunother. 38:92-98 (1994)). Similarly, other anthracyclins, including idarubicin and daunorubicin, have been chemically conjugated to antibodies, which have delivered effective doses of the agents to tumors (Rowland et al., Cancer Immunol. Immunother. 37:195-202 (1993); Aboud-Pirak et al., Biochem. Pharmacol. 38:641-648 (1989)).

In addition to the anthracyclins, alkylating agents such as melphalan and chlorambucil have been linked to antibodies to produce therapeutically effective conjugates (Rowland et al., Cancer Immunol. Immunother. 37:195-202 (1993); Smyth et al., Immunol. Cell Biol. 65:315-321 (1987)), as have vinca alkaloids such as

vindesine and vinblastine (Aboud-Pirak et al., *supra*, 1989; Starling et al., Bioconj. Chem. 3:315-322 (1992)). Similarly, conjugates of antibodies and antimetabolites such as 5-fluorouracil, 5-fluorouridine and derivatives thereof have been effective in treating tumors (Krauer et al., Cancer Res. 52:132-137 (1992); Henn et al., J. Med. Chem. 36:1570-1579 (1993)). Other chemotherapeutic agents, including cis-platinum (Schechter et al., Int. J. Cancer 48:167-172 (1991)), methotrexate (Shawler et al., J. Biol. Resp. Mod. 7:608-618 (1988); Fitzpatrick and Garnett, Anticancer Drug Des. 10:11-24 (1995)) and mitomycin-C (Dillman et al., Mol. Biother. 1:250-255 (1989)) also are therapeutically effective when administered as conjugates with various different antibodies. A therapeutic agent can also be a toxin such as ricin.

A therapeutic agent can also be a physical, chemical or biological material such as a liposome, microcapsule, micropump or other chambered microdevice, which can be used, for example, as a drug delivery system. Generally, such microdevices, should be nontoxic and, if desired, biodegradable. Various moieties, including microcapsules, which can contain an agent, and methods for linking a moiety, including a chambered microdevice, to an antibody of the invention are well known in the art and commercially available (see, for example, "Remington's Pharmaceutical Sciences" 18th ed. (Mack Publishing Co. 1990), chapters 89-91; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press 1988)).

For diagnostic purposes the antibody, or functional fragment thereof, can further comprise a detectable moiety. A detectable moiety can be, for example, a radionuclide, fluorescent, magnetic, colorimetric moeity, and the like. For *in vivo* diagnostic purposes, a moiety such as a gamma ray emitting radionuclide, for example, indium-111 or technitium-99, can be linked to an antibody of the invention and, following administration to a subject, can be detected using a solid scintillation detector. Similarly, a positron emitting radionuclide such as carbon-11 or a paramagnetic spin label such as carbon-13 can be linked to the molecule and, following administration to a subject, the localization of the moiety can be detected using positron emission transaxial tomography or magnetic resonance imaging, respectively. Such methods can identify a primary tumor as well as a metastatic lesion.

For diagnostic purposes, the antibodies of the invention can be used to determine the levels of denatured collagen in a tissue or in a bodily fluid. The level of denatured collagen can be determined in a tissue sample obtained from an individual, for example, by tissue biopsy. Exemplary bodily fluids include, but are not limited to, serum, plasma, urine, synovial fluid, and the like.

The invention also provides a method of inhibiting angiogenesis by administering an antibody, or functional fragment thereof, where the antibody or functional fragment thereof has specific binding activity for a cryptic collagen epitope, where the antibody

comprises one or more CDRs of the invention. For example, an antibody of the invention can be administered so that angiogenesis is inhibited in a tissue of an individual. The invention further provides a method of targeting a tumor by administering an invention antibody. The invention also provides a method of inhibiting tumor growth by administering an antibody, or functional fragment thereof, of the invention.

The antibodies of the invention can also be used for *in vivo* or *in vitro* diagnostic applications. Thus, the invention provides a method of detecting angiogenic vasculature by contacting angiogenic vasculature with an antibody, or functional fragment thereof, of the invention. Angiogenic vasculature can be imaged *in vivo* by administering an antibody of the invention, either alone or attached to a detectable moiety, to an individual. The angiogenic vasculature can thus be detected *in vivo*. Alternatively, the antibody can be administered to a tissue obtained from an individual, for example, a tissue biopsy, such that an antibody of the invention can be used *in vitro* for diagnostic purposes to detect angiogenic vasculature.

A therapeutic or detectable moiety can be coupled to an antibody of the invention, or functional fragment thereof, by any of a number of well known methods for coupling or conjugating moieties. It is understood that such coupling methods allow the attachment of a therapeutic or detectable moiety without interfering or inhibiting the binding activity of the antibody, that is, the ability to bind a cryptic collagen site. Methods for conjugating moieties to an antibody of

the invention, or functional fragment thereof, are well known to those skilled in the art (see, for example, Hermanson, Bioconjugate Techniques, Academic Press, San Diego (1996)).

When administered to a subject, the antibody of the invention is administered as a pharmaceutical composition containing, for example, the antibody and a pharmaceutically acceptable carrier. As disclosed herein, the antibody can be coupled to a therapeutic or detectable moiety. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art will know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition. The pharmaceutical composition also can contain an agent such as a cancer therapeutic agent.

One skilled in the art will know that a pharmaceutical composition containing an antibody of the invention can be administered to a subject by various routes including, for example, orally or parenterally, such as intravenously. The composition can be administered by injection or by intubation. The pharmaceutical composition also can be an antibody linked to liposomes or other polymer matrices, which can have incorporated therein, for example, a drug such as a chemotherapeutic agent (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed. (CRC Press, Boca Raton FL (1993), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

For diagnostic or therapeutic methods disclosed herein, an effective amount of the antibody and therapeutic moiety is administered to the subject. As used herein, the term "effective amount" means the amount of the pharmaceutical composition that produces the desired effect. An effective amount often will depend on whether the antibody itself is administered or whether the antibody is linked to a moiety and the type of moiety. Thus, a lesser amount of a radiolabeled molecule can be required for imaging as compared to the amount of a radioactive drug/antibody conjugate administered for therapeutic purposes. An effective amount of a particular antibody/moiety for a specific purpose can be determined using methods well known to those in the art. One skilled in the art can readily determine an

appropriate dose of an antibody of the invention for an effective amount for therapeutic or diagnostic purposes.

For therapeutic or *in vivo* diagnostic purposes, it is understood that any of a variety of methods of administration can be used so long as the administration is effective for a desired purpose. Such methods of administration include, for example, intravenous, transdermal, intrasynovial, intramuscular, intratumoral, intraocular, intranasal, intrathecal, topical, oral, or the like. One skilled in the art can readily determine an appropriate mode of administration depending on the desired therapeutic effect or desired diagnostic purpose.

Furthermore, it is understood that for therapeutic or diagnostic applications, an antibody of the invention in general is administered to a mammal, for example, a human. Applications of an antibody of the invention for domestic animals or agricultural purposes include other mammals, for example, a non-human primate, pig, cow, horse, goat, sheep, mule, donkey, dog, cat, rabbit, mouse, rat, and the like.

It is understood that any of the therapeutic methods disclosed herein using an antibody of the invention can be used in combination with other therapeutic methods. For example, an antibody of the invention, either the antibody itself or an antibody attached to a therapeutic agent, can be administered simultaneously or sequentially with other therapeutic treatment regimens. For example, an antibody of the invention can be administered alone or in combination with another therapeutic treatment, including any of the

therapeutic drugs disclosed herein as well as other drugs well known to those skilled in the art for treating a particular disease. For example, in the case of treating a cancer, an antibody of the invention can be administered simultaneously or sequentially with another chemotherapeutic agent such as a drug or radionuclide. Similarly, an antibody of the invention can be combined with other treatment regimens such as surgery by administering the antibody before, during or after surgery. One skilled in the art will know or can readily determine a desirable therapeutic treatment to be used in combination with an antibody of the invention, as desired. Thus, an antibody of the invention can be administered in conjunction with other therapeutic regimens, including but not limited to chemotherapy, radiation therapy, surgery, and the like.

The invention additionally provides a method of inhibiting metastasis using an antibody of the invention. The method can include the step of administering an antibody, or functional fragment thereof, having binding activity for a cryptic collagen epitope. The antibody can be, for example, an antibody comprising one or more CDRs having a least one amino acid substitution in one or more heavy or light chain CDRs of antibodies HUIV26 and HUI77. As used herein, inhibiting metastasis refers to decreasing the number and/or size of metastatic sites remote from a primary tumor site. The method of inhibiting metastasis can involve using an antibody of the invention that blocks adhesion of tumor cells to a cryptic collagen epitope that is exposed after remodeling of tissues by the action of collagen-degrading enzymes secreted by tumor cells.

As disclosed herein, a variant of HUI77 having one or more amino acid substitutions in one or more CDRs inhibited proliferation of melanoma cells *in vitro* (see Example VI). An antibody of the invention can block access to or inhibit binding of a survival or proliferative signal delivered to a tumor cell. Thus, the invention also provides a method of targeting a tumor cell by administration of an antibody of the invention having binding activity for a cryptic collagen epitope that blocks access to a survival or proliferative signal delivered to the tumor cell by a cryptic collagen site.

For methods of inhibiting angiogenesis, the angiogenic vasculature can be associated with a tumor. The methods of the invention can also be used to inhibit tumor growth directly, alone or in combination with inhibiting angiogenic vasculature of the tumor. The methods of the invention can additionally be used to inhibit metastasis, alone or in combination with inhibiting tumor angiogenic vasculature and/or tumor growth. Exemplary tumors include, but are not limited to, those disclosed herein, including melanoma, carcinoma, sarcoma, fibrosacroma, glioma, astrocytoma, and the like. Methods for testing the effect a HUIV26 or HUI77 variant for inhibition of angiogenesis or inhibition of tumor growth can be performed as described previously using, for example, assays such as the rat corneal micropocket angiogenesis assay, chick embryo tumor growth assay, or SCID mouse tumor growth assay, as described in Xu et al., *supra*, 2001, or any other well known assays for measuring inhibition of angiogenesis, inhibition of tumor growth, or inhibition of metastasis.

The methods of the invention can also be applied to inhibiting non-tumor angiogenic vasculature. Such applications to non-tumor angiogenic vasculature can include tissue that is inflamed and in which angiogenesis is occurring. Exemplary non-tumor diseases associated with angiogenic vasculature suitable for treatment with an antibody of the invention include, but are not limited to, those disclosed herein, including arthritis, ocular disease, retinal disease, hemangioma, and the like. The antibodies of the invention can also be used to inhibit psoriasis, macular degeneration, restenosis, and the like, or any tumor or non-tumor disease associated with increased accessibility of a cryptic collagen epitope for which an antibody of the invention has binding activity.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Cloning of Heavy and Light Chain Variable Regions of HUIV26 and HUI77 Antibodies

This example describes the cloning of HUIV26 and HUI77 antibody variable regions.

The variable regions of the HUIV26 and HUI77 antibodies were cloned from hybridomas expressing these mouse monoclonal antibodies and sequenced. Briefly, total mRNA was isolated from the respective mouse

hybridoma cells using Oligotex® Direct mRNA Micro kit (Qiagen; Valencia CA). First strand cDNA was synthesized from the mRNA using SuperScript Preamplification System (GibcoBRL/Invitrogen; Carlsbad CA). Antibody variable region sequences were amplified by PCR using a set of 5' primers designed for signal sequences of mouse light chains or heavy chains to pair with single 3' primer to mouse kappa chain constant region for V_L or IgM CH1 region for V_H sequences. The sequences of the 5' primers for the signal peptide of mouse antibody heavy and light chain as well as constant region primers are shown in Figure 1. The 3' primer for mouse kappa light chain constant region (primer 2650; SEQ ID NO:212) corresponds to amino acids 115-123. The 3' primer for mouse IgM CH1 region (primer 2656; SEQ ID NO:213) corresponds to amino acids 121-114. The 3' primer for mouse IgM CH1 region (primer 2706; SEQ ID NO:214) corresponds to amino acids 131-124.

The DNA fragments were isolated from PCR reactions, with a main product of about 400 bp in length. The DNA fragments were cloned into the pCR2.1 vector. The inserted DNA fragments were sequenced with both forward and reversed M13 primers. The DNA sequences were compared with an antibody sequence database. The N-terminal amino acid sequence of the HUIV26 and HUI77 antibodies were determined, and the sequences of the DNA fragments were also compared to the N-terminal amino acid sequences of the corresponding antibody.

The HUIV26 V_L encoding nucleic acid was cloned with 5' primer mK2 (primer 2664; SEQ ID NO:185) and 3' primer 2650 (SES ID NO:212). A partial sequence of

HUIV25 V_L is ATCTTCTTGCTGTTCTGG*GTATCTGGAACCTGTGGG* (SEQ ID NO:215), with the MK2 primer underlined and the partial sequence coding for mouse signal peptide in italics. The HUIV26 V_H encoding nucleic acid was cloned with 5' primer MH12 (primer 2731; SEQ ID NO:203) and 3' primer 2706 (SEQ ID NO:214).

The HUI77 V_L encoding nucleic acid was cloned with 5' primer mK1 (primer 2663; SEQ ID NO:184) and 3' primer 2650 (SEQ ID NO:212). A partial sequence of HUI77 V_L is TTGGTGCTGATGTTCTGG*ATTCCTGCTTCCAGCAGT* (SEQ ID NO:216), with the mK1 primer underlined and the partial sequence coding for mouse signal peptide in italics. The HUI77 encoding nucleic acid was cloned with 5' primers MH15 (primer 2734; SEQ ID NO:206) or MH16 (primer 2735; SEQ ID NO:207) and 3' primer 2656 (SEQ ID NO:213).

The sequences of the heavy and light chain nucleotide and amino acid sequences for HUIV26 and HUI77 are shown in Figures 2 and 3, respectively. Using the numbering system of Kabat, *supra*, the CDRs of the heavy and light chains were identified for each of the HUIV26 and HUI77 antibodies (underlined in Figures 2C and 3C).

An alignment of the HUI77 V_L nucleotide sequence (SEQ ID NO:9) with the nucleotide sequence of the human framework fusion DPK13/JK1 (SEQ ID NO:17) is shown in Figure 3D. The corresponding light chain amino acid sequences are referenced as SEQ ID NO:10 and SEQ ID NO:18 for HUI77 and DPK13/JK1, respectively.

This example describes the cloning and the sequence of mouse antibodies HUIV26 and HUI77.

EXAMPLE II

Generation of CDR Variant Libraries of HUIV26 and HUI77 Antibodies

This example describes the generation of CDR variant libraries of HUIV26 and HUI77 antibodies for CDR optimization.

The CDR3 regions of antibodies HUIV26 and HUI77 were optimized by generating a library of CDR variants. Primers for light chain CDR3 and heavy chain CDR3 were used to generate a library of CDR3 variants, where the primer was synthesized to encode more than one amino acid one or more positions in CDR3. Following synthesis of primers encoding CDR3 variants, the variant CDR3 regions were assembled into light chain (V_L) and heavy chain (V_H) regions.

Briefly, humanized V_L and V_H genes of HUI77 and HUIV26 antibodies were assembled with the primers shown in Figures 4A and 5A, respectively, using PCR or primer-elongation-ligantion. Variable region genes containing CDR3 mutations were assembled by replacing the wild type CDR3 primer (IV26-17, IV26-h7, I77-17 or I77-h7) with the group of mutant primers corresponding to that CDR. The assembled variable regions were then amplified and asymmetrically biotinylated on plus strand by PCR using primers B-pelB and 224 for V_L and B-phA and 1200a for H_v genes. The primers for amplification of humanized V_L and V_H sequences and the isolation of minus strand DNA were: B-pelB, Biotin-TTA CTC GCT GCC CAA CCA GCC ATG GCC (SEQ ID NO:220); 224, GAC AGA TGG TGC AGC CAC AGT (SEQ ID NO:221); B-phoA, Biotin-TTA CTG TTT ACC CCT GTG ACA AAA

GCC (SEQ ID NO:222); and 1200a, GAA GAC CGA TGG GCC CTT GGT (SEQ ID NO:223).

The assembled V_L and V_H regions were introduced into a Fab expression vector by mutagenesis. Briefly, the non-biotinylated minus strands were isolated after binding the PCR products to NeutrAvidin-conjugated magnetic beads and introduced into the Fab expression vector IX-104CSA by hybridization mutagenesis (Kristensson et al., Vaccines 95, pp. 39-43, Cold Spring Harbor Laboratory, Cold Spring Harbor (1995); Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492 (1985); Wu et al., J. Mol. Bio. 294:151-162 (1999)).

Three humanization-CDR3-mutation libraries were constructed for each the HUI77 and HUIV26 antibodies. The three libraries introduced random mutations but differed in CDR3 mutations. One library had mutations only in LCDR3, the second library had mutations only in HCDR3, and the third library had mutations in both LCDR3 and HCDR3.

Methods essentially the same as those described above for CDR3 mutagenesis were also performed on CDR1 and CDR2 of the HUIV26 and HUI77 antibodies. After assembling into a Fab expression vector, the Fabs containing HUIV26 and HUI77 variant CDRs were expressed in bacteria and tested for binding to denatured collagen. The mutant libraries were screened with filter lift screening and ELISA. The assays were performed essentially as described previously (Huse et al., J. Immunol. 149:3914-3920 (1992); Watkins et al., Anal. Biochem. 253:37-45 (1997)). Briefly, nitrocellulose

membranes were pre-coated with heat-denatured human collagen I or IV and used to lift *E. coli*-expressed variant FABs from phage plates. The membranes were then incubated with antibodies, either anti-human kappa chain or anti-hemaglutinin (HA) tag conjugated to alkaline phosphatase to detect bound variant Fabs. Positive clones were screened again by single point ELISA (Watkins et al., *supra*, 1997) for binding to denatured-biotinylated human collagen I and IV, correspondingly. Beneficial variants were characterized for binding to both collagens in native and heat-denatured forms by ELISA. Beneficial mutations were determined as those having higher affinity binding to denatured collagen relative to the corresponding wild type Fab, as demonstrated by ELISA.

Shown in Figures 4B and 5B is a summary of beneficial CDR mutations in the HUIV26 and HUI77 antibodies, respectively. Figure 4B summarizes beneficial single amino acid mutations in heavy chain CDR1, CDR2, and CDR3 and light chain CDR1 and CDR3 of HUIV26. An exemplary HUIV26 variant having a single amino acid substitution is the 12F10Q variant, which exhibited k_{on} of 0.055 and k_{off} of 0.049 as estimated by the fold improvement based on shifts in half-maximal binding obtained from ELISA titrations.

Figure 5B summarizes beneficial single amino acid mutations in heavy chain CDR1, CDR2 and CDR3 and light chain CDR1, CDR2 and CDR3 of HUI77. As can be seen, numerous single amino acid mutations in various CDRs were found to maintain or enhance binding to a cryptic collagen site.

This example describes CDR variants of HUIV26 and HUI77 having beneficial mutations.

EXAMPLE III

Identification of Combinatorial Variants of HUIV26 and HUI77 Antibodes Having Enhanced Activity

This example describes the generation and identification of combinatorial variants incorporating various beneficial CDR mutations in HUIV26 and HUI77.

To further optimize HUIV26 and HUI77 antibody CDR variants, combinatorial variants, which incorporate at least two CDRs containing one or more mutations, were generated and tested for binding to a cryptic collagen site. Combinatorial variants were synthesized using primers with one or more positions encoding variant amino acids as described in Example II. The primers used are shown in Figures 6 and 7.

Shown in Figures 6 and 7 is a summary of the beneficial combinatorial variants of HUIV26 and HUI77 antibodies, respectively. The k_{on} and k_{off} values shown in Figures 6 and 7 ("SPEKon" and "SPEkoff") were estimated as the fold improvement of variants based on shifts in half-maximal binding obtained from ELISA titrations. Also shown are several variants having the same beneficial CDR mutations but having different framework sequences. These results show that beneficial CDR mutations can be grafted into a variety of frameworks and can retain or have improved binding activity.

This example shows the generation of combinatorial CDR variants of HUIV26 and HUI77. A number of variants were identified having increased affinity relative to wild type forms of the respective antibodies.

EXAMPLE IV

Binding Activity and Specificity of HUIV26 and HUI77 Variants

This example describes the binding activity and specificity of HUIV26 and HUI77 antibodies on native and denatured collagen.

The activity and specificity of wild type and selected exemplary HUIV26 and HUI77 variants were determined. As shown in Figure 8, the activity and specificity of IX-IV26, a Fab containing wild type HUIV26 CDRs, and the HUIV26 variants 2D4H1-C3 and DhuG5 were determined. The antibodies were tested for binding to denatured collagen IV (Figure 8A), denatured collagen I (Figure 8B), and native collagen IV (Figure 8C). None of the antibodies had significant binding activity for native collagen IV (Figure 8C). All three antbodies exhibited binding activity for denatured collagen IV (Figure 8A). However, the 2D4H1-C3 and DhuG5 variants exhibited significantly increased binding activity relative to IX-IV26 (Figure 8A). IX-IV26 did not exhibit significant binding activity to denatured collagen I, and 2D4H1-C3 and DhuG5 exhibited low binding activity at the highest measured concentration of antibody (Figure 8B). These results indicate that the HUIV26 variants have similar binding activity and specificity as that of wild type HUIV26 and maintain activity and specificity for a

cryptic collagen epitope. These results further show that variants having mutated CDRs can have maintained or increased binding affinity relative to wild type.

As shown in Figure 9, the activity and specificity of IX-I77, a Fab containing wild type HUI77 CDRs, and the HUI77 variants Qh2b-B7 and QhuD9 were determined. The antibodies were tested for binding to denatured collagen I (Figure 9A), denatured collagen IV (Figure 9B) and native collagen I (Figure 9C), and the results indicate that these variants exhibited similar binding specificities as wild type. Neither IX-I77 nor Qhu2b-B7 exhibited significant binding activity for native collagen I, although the variant QhuD9 exhibited modest binding activity to native collagen at higher concentrations of antibody. The antibodies all exhibited binding activity for denatured collagen I (Figure 9A) and denatured collagen IV (Figure 9B). However, the Qhu2b-B7 and QhuD9 variants exhibited significantly increased binding activity relative to IX-I77 on both denatured collagen I and IV. These results indicate that variants having mutated CDRs can have maintained or increased binding affinity relative to wild type.

To further examine the effect of CDR mutations on binding activity, the HUIV26 variant DhuH8 was selected and expressed in two forms, as a Fab and immunoglobulin (IgG). The binding activity of these two forms was determined for native (n-IV) and denatured (d-IV) human collagen IV. As shown in Figure 10, neither the Fab nor IgG form of the Dhu8 variant exhibited significant binding to native collagen IV. The Fab form exhibited binding activity for denatured collagen IV, and

the binding affinity was significantly increased for the IgG form. These results indicate that a HUIV26 variant having one or more CDR amino acid substitutions relative to wild type can exhibit binding to a cryptic collagen epitope and that the binding affinity can be significantly increased in the IgG form relative to the Fab form of the antibody variant.

These results indicate that HUIV26 and HUI77 variants having one or more CDR amino acid substitutions can exhibit similar binding specificity and increased binding affinity relative to wild type.

EXAMPLE V

Generation of Grafted HUIV26 and HUI77 Antibodies Having Optimized CDRs

This example describes the generation of humanized HUIV26 and HUI77 antibodies incorporating beneficial CDR mutations.

A CDR variant have a beneficial mutation is identified as described in Examples II and III. Once a beneficial CDR variant is identified, the CDR variant is grafted into a human framework sequence. In addition to the CDR variant having a beneficial mutation, other CDRs can be a wild type sequence of the respective antibody or one or more variant CDRs. At least one of the CDRs will be a variant containing a beneficial mutation. For example, if the grafted antibody contains a heavy and light chain, at least one of the heavy or light chain CDRs will have at least one amino acid mutation relative to the corresponding wild type CDR.

A human framework sequence is selected as the recipient for grafting. The human framework can be closely related to the donor antibody framework sequence or can be relatively divergent from the parental donor antibody. Once a human framework is selected for grafting, overlapping oligonucleotides are synthesized encoding the selected human framework and the appropriate donor CDRs, including at least one variant CDR containing at least one beneficial mutation. The overlapping oligonucleotides are used to assemble a nucleic acid encoding a variable region including the selected human framework, the CDR variant, and appropriate other CDRs to generate an antibody or fragment having binding activity for a cryptic collagen site.

The assembled variable region is cloned into an expression vector, for example, a Fab expression vector such as described in Example II, and binding activity to denatured collagen is tested, as described in Examples II and III.

This example describes the generation of humanized antibodies containing beneficial CDR mutations of HUIV26 and HUI77 antibodies.

EXAMPLE VI

Inhibition of B16 Melanoma Cell Proliferation by a Variant HUI77 Antibody

This example describes the effect of the HUI77 variant QH2b on B16 melanoma cell proliferation.

The humanized Fab designated QH2b, which is the QH2b-B7 variant of the HUI77 antibody, was engineered into a full length IgG1 antibody (QH2b-IgG1). The QH2b-IgG1 antibody was expressed in mammalian cell culture in NSO cells and purified.

The purified QH2b-IgG1 antibody was used in a cell proliferation assay *in vitro*. B16 melanoma cells were plated on denatured human Type I collagen. QH2b-IgG1 (100 µg/ml/day) was added to one set of culture dishes and cell numbers were determined at the indicated times (Figure 11). As a control, the cells were not treated with antibody.

As shown in Figure 11, B16 melanoma cells proliferated on denatured collagen type-I, as indicated by the increase in cell numbers over 3 days. The B16 melanoma cell cultures treated with QH2b-IgG1 exhibited essentially no cell growth over a period of 3 days, indicating that the melanoma cells did not proliferate in the presence of the HUI77 variant QH2b-IgG1.

These results indicate that a HUI77 variant having one or more CDR amino acid substitutions can inhibit cell proliferation of B16 melanoma cells.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above,

it should be understood that various modifications can be made without departing from the spirit of the invention.